DEVELOPMENT OF MHC CLASS II-RESTRICTED TCR GENE THERAPY FOR EPSTEIN BARR VIRUS ASSOCIATED MALIGNANCIES

By

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A thesis submitted to

The University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

The School of Cancer Sciences

College of Medical and Dental Sciences

The University of Birmingham

September 2015

ABSTRACT

CD4+ T-cells play a pivotal role within the immune response, and multiple studies have highlighted their importance in anti-tumour immunity. TCR gene transfer is a successful method of specifically redirecting T-cell specificity. We have therefore investigated the anti-tumour potential of EBV-specific MHC class II restricted T-cells, generated by this approach. We have identified and cloned a DR52b-restricted TCR, specific for an EBNA2 derived peptide (PRS), which is expressed in Post-Transplant Lymphoproliferative Disease (PTLD) and some other EBV-associated malignancies.

We have shown that the TCR is functional in both CD4+ and CD8+ T-cells, with transduced T-cells specifically recognising the PRS-peptide with a high avidity. Transduced T-cells have been shown to proliferate, produce multiple cytokines and have direct cytotoxic capacity in response to physiological levels of EBNA2 processed and presented by EBV-infected B-cells. Additionally to this direct response, CD4+ T-cells retain helper functions. Importantly, transduced T-cells have shown hints of tumour control *in vivo.*

Results from this study highlight that TCR gene transfer with EBV-specific MHC class II-restricted TCRs can generate polyclonal T-cells with functional capacity against virus-infected cells. PRS specific TCR gene transfer may thus be useful in rapid generation of T-cells for treatment of PTLD. Given the importance of CD4+ T-cells for anti-tumour responses, this study also highlights the potential for using TCR gene transfer to target these cells towards other MHC class II-positive tumours.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors Steve and Heather for the help and guidance provided over the last four years. I appreciate your open doors and believe I would not have got here without your support. Heather, your patience with me as a new student was admirable, I'll do my best to follow this example in my future career. Steve, the countless hours we spent discussing science in your office were invaluable, and I'm glad for every minute (and apologise if I didn't always seem it)! I would also like to thank all the members of the Lee and T-cell group for the help and advice given throughout my time here. In addition I would like to thank everyone who donated blood – with special thanks to one person in particular – you know who you are. Thanks to Eszter Nagy for help with Immunohistochemistry and Maha Ibrahim for analysis of data produced.

Special thanks go out to all of my family and friends – along with giving advice when needed, your happy faces (and thesis cakes – a personal highlight) kept me sane through writing up and for that I'm really grateful. Kristina - I mention you here as I think you'll read this thesis start to finish - your office giggle is something I'll really miss!

And finally Ben. Eternal thanks for proof reading those three pages (and, you know, being my rock these last four years).

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LIST OF ABBREVIATIONS

Abbreviation	Word
ACT	Adoptive cell therapy
ADCC	Antibody dependent cell-mediated cytotoxicity
ALL	Acute lymphocytic leukaemia
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-7
APCs	Antigen presenting cells
BARTs	BamHI A rightward transcripts
BCR	B-cell receptor
BL	Burkitt's lymphoma
C	Constant
CAR	Chimeric antigen receptor
CDR	Complementarity determining region
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMPs	Danger Associated Molecular Patterns
DCs	Dendritic cells
DLBCL	Diffuse large B-cell lymphoma
DLI	Donor lymphocyte infusion
DMEM	Dulbecco's Modified Eagle Medium
E	Early

EBERs	EBV encoded RNAs
EBNA	EBV nuclear antigen
EBV	Epstein Barr Virus
ECD	Electron coupled dye
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
FasL	Fas ligand
FFPE	Fixed frozen paraffin embedded
FITC	Flourescein Isothicyanate
GvHD	Graft versus host disease
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HPCs	Hematopoietic progenitor cells
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
HSV TK	Herpes Simplex Virus Thymidine Kinase
IE	Immediate early
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL12	Interleukin 12
IL2	Interleukin 2
IL6	Interleukin 6
IM	Infectious mononucleosis

IP	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
J	Joining
JNK	c-Jun N-terminal Kinase
L	Late
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
MART 1	Melan A
MDSCs	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
MR1	MHC related protein 1
mRNA	Messenger ribonucleic acid
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa light chain enhancer of activated B-cells
NK	Natural killer
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PD1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PE	Phycoerythrin
PE-Cy7	Phycoerythrin cyanine 7
p-MHC	Peptide-MHC
PRR	Pattern recognition receptor
PTLD	Post transplant lymphoproliferative disease

RAG	Recombination activation gene
RNAi	Ribonucleic acid interference
ScFV	Single chain variable fragment
SMAC	Supramolecular activation cluster
SOC	Super optimal broth
SOT	Solid organ transplant
Tcm	Central memory T-cells
TCR	T-cell receptor
TECs	Thymic epithelial cells
Teff	Effector T-cells
Tem	Effector memory T-cells
TIL	Tumour infiltrating lymphocyte
Tmem	Memory T-cells
TPA	Tetradecanoyl phorbol acetate
Tregs	Regulatory T-cells
Tscm	Stem cell memory T-cells
TRUCKS	T-cells redirected for universal cytokine mediated killing
uNPCs	Undifferentiated nasopharyngeal carcinoma
V	Variable
WHO	World health organisation
WT1	Wilms tumour gene 1

CHAPTER 1

1 INTRODUCTION

That the immune system is naturally capable of recognising and eliminating tumours was first proposed just over 100 years ago by Paul Ehrlich [1]. From this evolved the cancer immunosurveillance hypothesis in 1957, when Burnett and Thomas identified spontaneous regression of syngeneically transplanted tumours in mice [2]. This hypothesis stated that the immune system could destroy cancer cells, providing they displayed an altered antigenic repertoire. Then followed the notion that the immune system can be enhanced to improve the anti-cancer response. In fact, William Coley first attempted to treat malignancies using the immune system in 1893 by stimulating the host's immune system after observing cancer regression in a patient who experienced multiple bacterial infections [3]. Immunotherapy for cancer is now a rapidly growing area of clinical research, with numerous reports of successful trials of immunotherapy in multiple cancer types. This growth is due to the technological advancements within immunology and molecular biology fields. Indeed, highlighting the success of this area was the declaration by the journal Science that Cancer Immunotherapy was the Breakthrough of the Year 2013.

1.1 The Human Immune System

As immunotherapies are based upon the immune system, we must have knowledge of this system prior to designing an effective immunotherapy. The immune system comprises the innate and adaptive systems and these will be discussed individually below.

1.1.1 Innate Immunity

The purpose of the innate response is to respond rapidly to invading pathogens. There are multiple components of the innate immune response, including epithelial barriers, anti-microbial substances and blood proteins, such as the complement system. Additionally, the innate immune response includes phagocytic leukocytes such as eosonophils, neutrophils, basophils and macrophages, dendritic cells (DCs) and the cytotoxic leukocytes natural killer (NK) cells [4]. These 'first-on-scene' cells respond quickly to shared features of pathogens (pathogen associated molecular patterns; PAMPs) or damaged self-cells (danger associated molecular patterns; DAMPs) [5]. Innate cells recognise these PAMPs and DAMPs through pattern recognition receptors such as Toll Like Receptors, and upon receptor binding, innate cells are stimulated to phagocytose or lyse target T-cells and pathogens and to activate an inflammatory response [6]. Phagocytic leukocytes phagocytose pathogens as well as infected or damaged cells. DCs process and present antigenic material to adaptive immune cells (see below), priming them for response [7]. NK cells recognise signals associated with aberrant cells such as MHC class I down-regulation and up-regulation of MHC class I chain related (MIC) molecules MICA and MICB. They respond by killing target T-cells and releasing cytokines [8].

1.1.2 Adaptive Immunity

The innate response is stimulated almost instantly after pathogenic infection. Whilst responding directly to the pathogen, this response also activates the adaptive immune response. This allows both systems to work in synergy to respond effectively to primary infection and to protect the body from subsequent invasions. Adaptive immune cells include

B- and T-cells. Each cell is tailored to target a specific antigen and thus for an effective response to be generated, the B- and T-cells which recognise the antigens present must first undergo activation and clonal expansion. Therefore the adaptive response is a delayed one. Once activated, these cells directly kill or induce the killing of infected cells. Target specific B-cells produce and secrete antibodies and cytokines in response to infection. Antibodies function by binding to their targets and inducing phagocytosis of the target T-cell and by antibody dependent cell-mediated cytotoxicity (ADCC). Following adaptive responses to infection, a proportion of B- and T- cells will differentiate into memory cells. Memory cells are maintained at a higher precursor frequency than their original parent clone and are able to function more rapidly upon re-encounter with target antigens. As such they provide a more rapid and magnified immune response upon secondary exposure to that antigen. As this thesis focuses on T-cell-based therapies for cancer, I shall focus on this immune cell type herein.

1.1.3 T-cells

1.1.4 T-cell receptors

T-cells recognise processed target antigens presented at the cell surface in Major Histocompatability Complex (MHC) molecules through their T-cell Receptors (TCRs). They respond by killing the cells and/or producing cytokines to activate a broad immune response. As T-cells recognise targets solely through their TCRs, the generation of TCRs is a critical step in T-cell development.

There are two main subtypes of T-cells, distinguished by the type of TCR genes expressed. T-cells can express TCRs generated from gamma and delta (γδ), or alpha and beta (αβ) TCR genes. All TCR chains are generated through a series of somatic gene rearrangements. The α

chain genes are generated by the rearrangement of DNA at the TCRα loci, which contain multiple variable (V), joining (J) and constant (C) gene segments. The TCRβ loci consists of these V, J and C gene segments but also has multiple diversity (D) gene segments, which increase the heterogeneity of the TCRβ chains (Figure 1.1) [9]. Somatic rearrangement results in the joining of one of each gene segment to form complete α and β chain genes. The number of V, J, D and C gene segments make TCRs highly heterogeneous. Random nucleotide insertion or mutation between the V, D and J gene segments further increases this heterogeneity. The most variable regions in the TCR chains are referred to as complementarity determining regions (CDRs 1, 2 and 3), with CDR3 being the most variable of all. It is these variable regions that determine the antigenic specificity of the TCR [10].

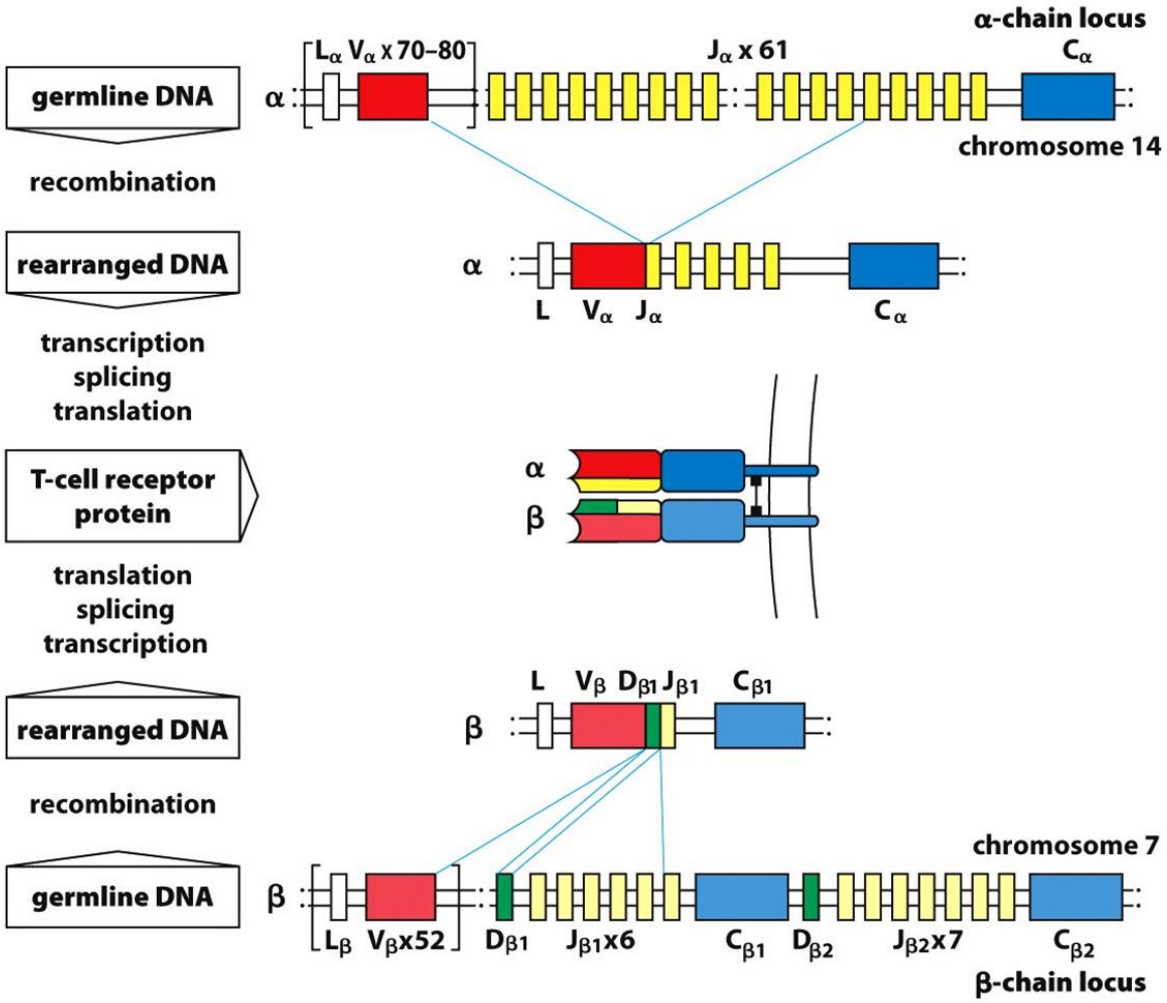


Figure 1.1 TCR gene rearrangement.

Alpha and Beta TCR genes are somatically rearranged in the thymus. Taken from The Immune System, 3ed. (© Garland Science 2009).

Each TCR has six highly variable CDRs located in the membrane-distal area of the TCR, where it interacts with peptide presented in MHC (p-MHC). TCRs also have more conserved areas, which form interactions with the MHC – which in humans is also termed Human Leukocyte Antigen (HLA) (Figure 1.2) [11].

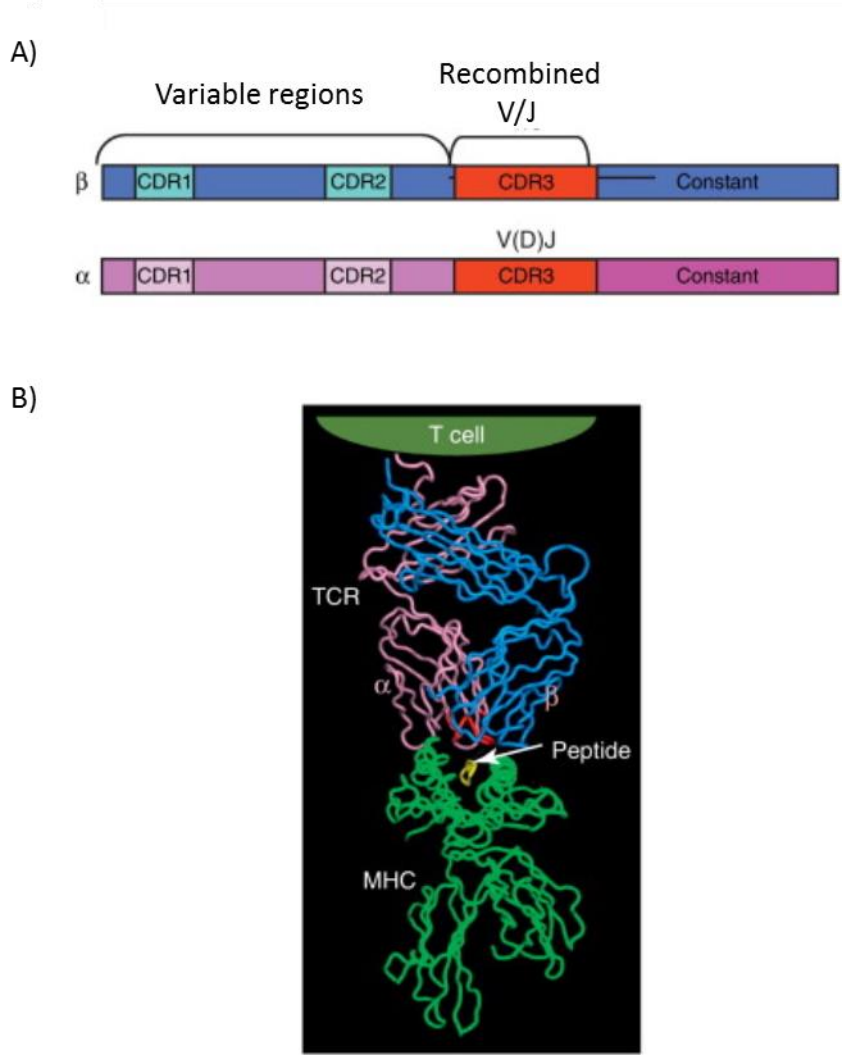


Figure 1.2 Diagram of TCR p-MHC interaction.

The alpha and beta chain of the TCR bind to the p-MHC complex, with the CDRs making the most interactions. A) Schematic view of alpha and beta TCR chains with colour coded CDRs B) illustration of how the TCR binds to p-MHC. The colours are in accordance to A). Adapted from [12]

TCR heterogeneity theoretically allows for the generation of approximately 10^{18} unique TCRs [13]. However, this is an overestimation, as some gene segments are not found in combination with each other. Furthermore, the number of TCRs in any one human is lower than this as the majority of TCRs are unable to pass successfully through central and peripheral tolerance (described below). Therefore, it is estimated that the average number of unique TCRs in one individual is $25x10^6$ [14]. This large TCR repertoire enables recognition of a vast number of epitopes, allowing the host to respond to the array of foreign pathogens and antigens encountered through the course of life.

1.1.5 TCR subtypes

Of the two main subtypes of T-cells, γδ TCRs recognise non-peptide targets such as pyrophosphate antigens and lipids, and reside mainly in tissues such as the gut and skin [15]. As many γδ TCR targets are expressed on tumour cells, this subset is under investigation for its immunotherapeutic potential. The majority of TCRs expressed on T-cells in the peripheral blood are generated from α and β TCR genes, and recognise peptides presented on MHC. A small proportion of αβ TCRs recognise glycolipids presented through MHC-like CD1 proteins, or bacterial metabolites, presented through MHC-related protein 1 (MR1) [16-18]. This thesis focuses on αβ TCRs which recognise peptide presented in MHC complexes, and therefore this will be explained in greater detail.

1.1.6 Antigen presentation

Antigen Presenting Cells (APCs) and target T-cells present antigen through MHC. Every individual inherits up to fourteen MHC (HLA) genes. HLA alleles are highly polymorphic, with approximately 9500 MHC class I alleles and 3000 MHC class II alleles identified as of

April 2015 (see http://hla.alleles.org/). Each allele can present a range of peptides, providing the peptides are the correct size and sequence to bind the peptide binding groove. The number of potential antigens that could be presented through MHC class I or MHC class II greatly exceeds the number of TCRs in the human repertoire. Therefore, T-cells have evolved to be able to recognise multiple peptides of similar shapes, due to TCR flexibility [19]. This cross reactivity allows high coverage of potential non-self antigens [13].

1.1.7 MHC class I antigen presentation

MHC class I genes are ubiquitously expressed in nucleated cells and present endogenous antigen to CD8+ T-cells. Endogenous proteins are degraded in the cytoplasm by proteasomes and transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) protein. Here the chaperone protein tapasin directs peptides into the MHC class I peptide binding grove. This p-MHC complex can then be transported to the cell surface.

It is also possible for MHC class I to present exogenous antigen acquired by the cell to CD8+ T-cells through cross priming [20].

1.1.8 MHC class II presentation of exogenous antigen

MHC class II alleles are constitutively expressed on professional APCs (DCs, monocytes and B-cells), thymic epithelium and activated T-cells, and can be expressed on other cell types when stimulated by interferon gamma (IFNγ) [21, 22]. In general, MHC class II presents exogenous antigen to CD4+ T-cells.

Through endocytosis, macropinocytosis and phagocytosis, proteins are taken up by APCs and enter cytosolic vesicles. Here, proteins are degraded into peptides by proteinases.

MHC complexes are translated in the endoplasmic reticulum (ER) and transported into the golgi by the aid of the chaperone protein, invariant chain (Ii). This binds to the MHC complex in the peptide binding groove in order to prevent MHC associations with peptides present in the ER [23]. From the golgi, they are sorted into endosomes and lysosomes which contain the degraded exogenous proteins [24]. Ii is then proteolytically degraded, leaving a small fragment (The Ii-derived class II invariant chain peptide (CLIP)) in the peptide binding groove [25]. This is removed in conditions of low pH by the class II -like chaperone HLA-DM. DM facilitates peptide exchange by stabilising the empty MHC class II complex [26]. Depending on their amino acid sequence, peptides are then able to bind to the MHC class II peptide-binding groove, and p-MHC complexes are transported to the cell surface for interaction with CD4+ T-cells (Figure 1.3).

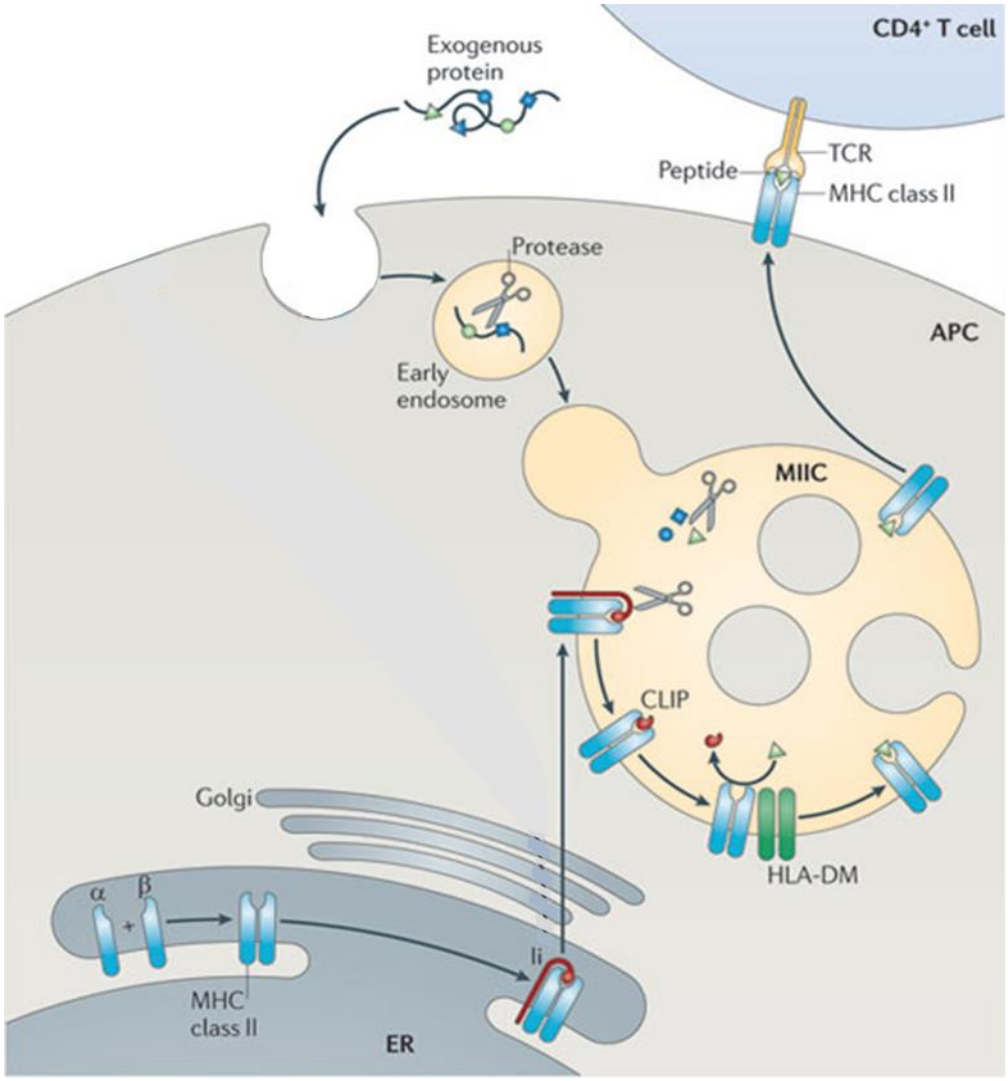


Figure 1.3 Exogenous antigen is processed and presented to CD4+ T-cells in MHC class II complexes.

MHC class II is assembled in the ER and translocates through the Golgi to endosomes containing exogenous proteins. Here, Invariant chain is removed from the peptide binding groove and replaced by foreign peptides. The peptide-MHC complex is directed to the plasma membrane for surface expression. Figure taken from [27].

1.1.9 MHC class II presentation of endogenous antigen

In some cases, endogenous antigen derived from nuclear or cytoplasmic proteins can also be processed for presentation to CD4+ T-cells through MHC class II [28]. In fact, up to 20% of antigens presented in MHC class II are derived from endogenous proteins [29]. Cells that express MHC class II can thus directly present endogenous proteins to CD4+ T-cells, providing these proteins enter the MHC class II processing pathway.

Endogenous antigens derived from self, tumour and viral proteins have been shown to access this pathway through different mechanisms. Firstly, endogenous proteins that naturally reside in the ER can be identified in MHC class II complexes [30]. Autophagy is another mechanism that results in MHC class II presentation of endogenous cytoplasmic (and less efficiently nuclear) antigens [29, 31-33]. Autophagy is the process of the cell sequestering part of it's cytoplasm in vesicles. These fuse to lysosomes, thereby transferring cellular contents to the same location as MHC class II [33]. Additionally, plasma membrane proteins that are internalised from the cell surface can enter endosomes. Here they are degraded and resulting peptides can bind to MHC class II [34]. Finally, if the infected or diseased cell is an APC, it can phagocytose neighbouring infected or diseased cells, or indeed uptake released antigen. This intercellular antigen transfer allows for cross presentation of antigens to CD4+ T-cells [35].

1.1.10 T-cell development

Before T-cells can respond to cells presenting their cognate p-MHC complexes, they must develop into functional naïve cells. T-cells are derived from hematopoietic progenitor cells

(HPCs). These cells are produced in the bone marrow and those which migrate to the thymus undergo T-cell maturation to develop into mature, naïve T-cells.

Through T-cell maturation T-cells differentiate into one of two lineages, and are distinguished by the presence of co-receptors CD4 and CD8. Following their identification, these subsets were attributed different functional characteristics. However following further investigation it is now clear that CD4+ and CD8+ T-cells are capable of producing similar functional responses, albeit with different efficiencies. Following priming by DCs (discussed below), CD8+ T-cells recognise target T-cells presenting antigen. This stimulates a cytotoxic effector response, resulting in target T-cell killing. CD4+ T-cells recognise APCs presenting cognate antigen and respond by producing cytokines to provide "help" to CD8+ T-cells and other arms of the adaptive and innate immune system. CD8+ T-cells have classically been considered the T-cell subset that mediates anti-tumour responses. However the role of CD4+ T-cells in this setting, and indeed the role of individual CD4+ subtypes, has been shown to be much more significant than originally considered.

During T-cell maturation, hematopoietic precursors enter the thymus and develop as they pass through the outer cortex, inner cortex and finally into the medulla [36]. T-cell maturation involves the expression of genes required for function such as TCR and co-receptor genes, and selection of those T-cells which are both functional and not auto-reactive.

Initially in T-cell maturation, no CD4 or CD8 co-stimulatory molecules are expressed (double negative T-cells). Expression of CD4 and CD8 ensues and these double positive T-cells interact with thymic cortical epithelial cells (TECs) which express self-antigens through MHC class I and II [37]. TECs express the transcription factor AIRE. This transcription factor induces the expression of genes which are normally expressed in a tissue dependent manner,

to ensure that mature T-cells are tolerant to antigens they will contact throughout the body [38]. Maturation into CD8 or CD4 single positive T-cells depends on whether the cells recognise antigens presented in MHC class I or II complexes, respectively. Through the process of positive selection, all T-cells which bind to MHC class I or II receive a survival signal. T-cells which do not bind receive no such signal and enter apoptosis [39]. Single positive T-cells are next subjected to negative selection in the medulla. Here, CD4+ or CD8+ T-cells interact with thymic epithelial cells expressing self-antigen. Any T-cells which bind to self-antigens with a high avidity receive an apoptosis signal and are deleted, or mature into regulatory T-cells (Tregs) [40]. Cells that survive both rounds of selection in the thymus leave the thymus to circulate the periphery as mature, naïve T-cells. Here, T-cells are subjected to peripheral tolerance. If a T-cell recognises a self-antigen that is not expressed in the thymus, in the absence of co-stimulation it will become tolerised and thus unable to respond to the presenting cell [41].

1.1.11 T-cell target priming

Naïve T-cells are primed for action upon encounter with professional APCs – such as DCs. DCs uptake antigen in infected tissues and migrate to lymph nodes. Here, they present antigen to naïve T-cells and provide the co-stimulation required for T-cell activation (see below). Primed CD8+ T-cells will then mature into effector cells and leave the lymph nodes to encounter and respond to target T-cells. Primed naïve CD4+ T-cells will differentiate into different T-cell subsets with different effector functions, depending upon the surrounding environment at the time of activation (Figure 1.4) [42]. This differentiation drives T-cells to perform a diverse array of immunological functions. As well as inducing differentiation, T-cell priming results in up-regulation of activation markers and an enhanced potential for

cytokine production and proliferation in CD4+ and CD8+ T-cells [43]. Together, this ensures that T-cells are ready to respond following further encounters with their cognate antigen.

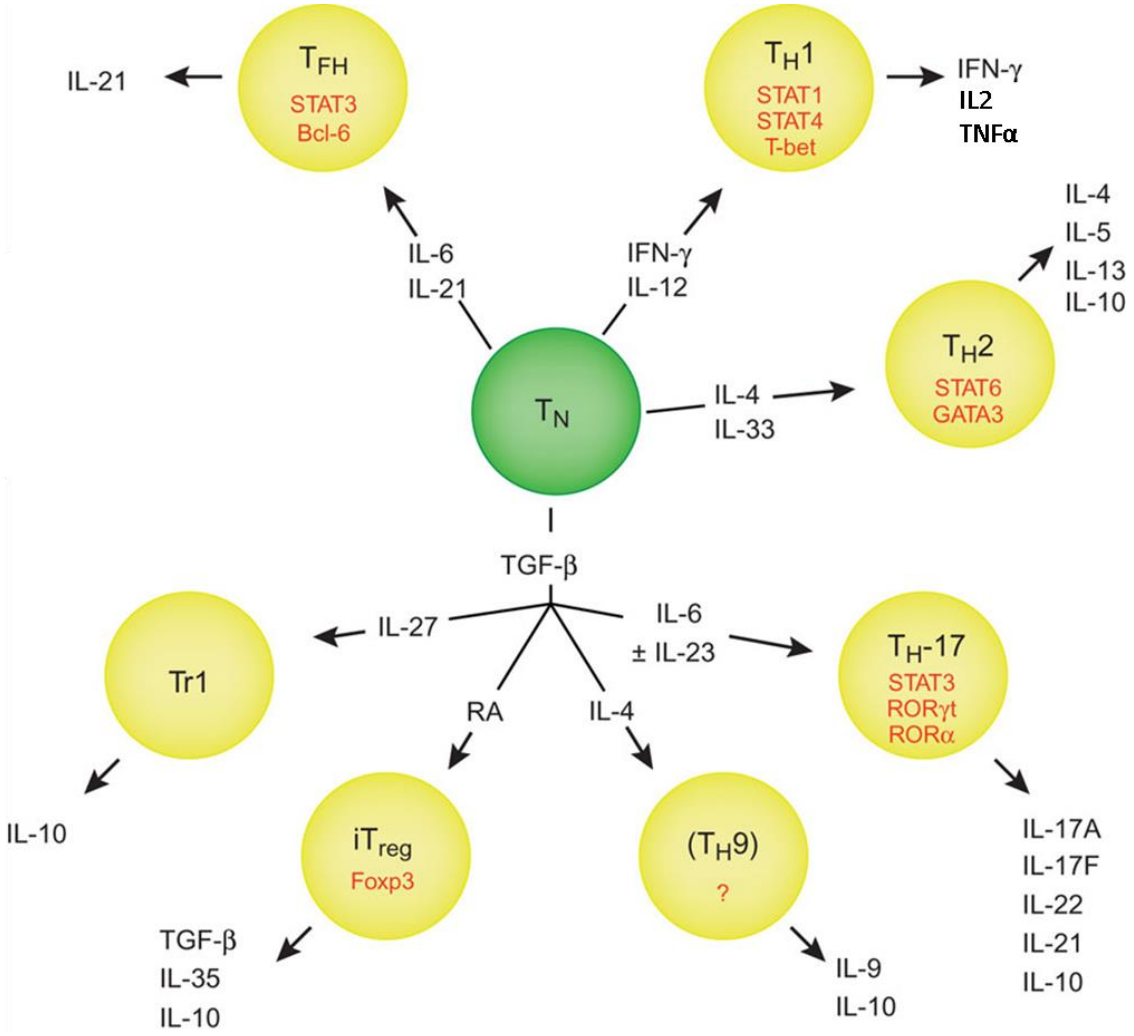


Figure 1.4. CD4+ T-cell subtypes.

Naive T-cells differentiate into different T-cell subtypes depending on the conditions in which they were primed. Different cytokines drive the activation of different transcription factors, which are responsible for the gene expression changes that drive T-cell differentiation. T-cell subtypes have differing effector functions. Adapted from [44].

1.1.12 T-cell activation

When T-cells form interactions with target T-cells presenting their cognate antigen after being primed for response, they become activated to respond.

T-cell activation involves a huge number of proteins translocating to the area of the plasma membrane which is closest to the target T-cell to form an immunosynapse. These proteins work in synergy in the T-cell/target T-cell immunosynapse, to allow for recognition and response to antigen (Figure 1.5). The most central region of the immunosynapse contains TCR complexes. These complexes consist of TCRs and CD3 co-receptor proteins, which are essential for T-cell activation. Additionally, co-receptors CD4 and CD8 form part of the TCR complex. When a TCR interacts with its target p-MHC complex, the CD4 or CD8 co-receptor will interact with the MHC allele, in order to potentiate antigen driven stimulation by stabilising interactions and recruiting signalling molecules [45].

For T-cell responses, TCRs need to bind to specific p-MHC complexes and co-stimulation must be present. Co-stimulation is required to enhance cytokine production in response to T-cell activation, increase proliferation and prevent anergy (which will render the cell unresponsive to secondary antigen encounters) [46, 47]. CD28 is a potent co-stimulatory molecule. It is expressed on T-cells and binds to CD80 or CD86 on target T-cells. Binding results in downstream T-cell signalling pathways to enhance T-cell activation [48]. Activated T-cells then upregulate surface expression of the co-inhibitory molecule cytotoxic T-lymphocyte associated protein 4 (CTLA-4). CTLA-4 is a CD28 homologue that binds to CD80 and CD86 with a tenfold higher affinity than CD28, and signals to inhibit T-cell activation [49]. This provides a negative feedback loop to prevent over-activation. Whilst

there are other co-stimulatory molecules, they are beyond the scope of this thesis and will not be discussed here.

Providing the TCR is specific for the p-MHC complex bound and the T-cell receives co-stimulation, cytoskeletal rearrangements allow for the directed translocation of multiple signalling molecules required for cell-cell adhesion and T-cell activation. This results in a supra-molecular activation complex (SMAC), in which numerous TCRs and costimulatory molecules reside [50]. When TCRs bind to their target p-MHC, conformational changes in CD3 result in the phosphorylation of CD3 immunoreceptor tyrosine based activation motifs (ITAMs). These phosphorylated regions provide docking and interaction sites for downstream src-related signalling molecules Lck and Fyn. These molecules activate the protein tyrosine kinase ZAP-70, in order to amplify the signalling response [51]. These interactions lead to the activation of a wide variety of downstream pathways, including the Extracellular Signal Regulated Kinase (ERK) and c-Jun N-terminal Kinase (JNK) pathways [51]. Additionally, signalling leads to calcium influx which, with the signalling pathways, results in the translocation of transcription factors such as Nuclear Factor kappa-light-chain-enhancer of activated B-cells (NFκB) and Nuclear Factor of Activated T-Cells (NFAT) to the nucleus. These transcription factors initialise transcription of many T-cell activation related genes. This regulates effector functions such as proliferation, differentiation and cytokine production etc. [52].

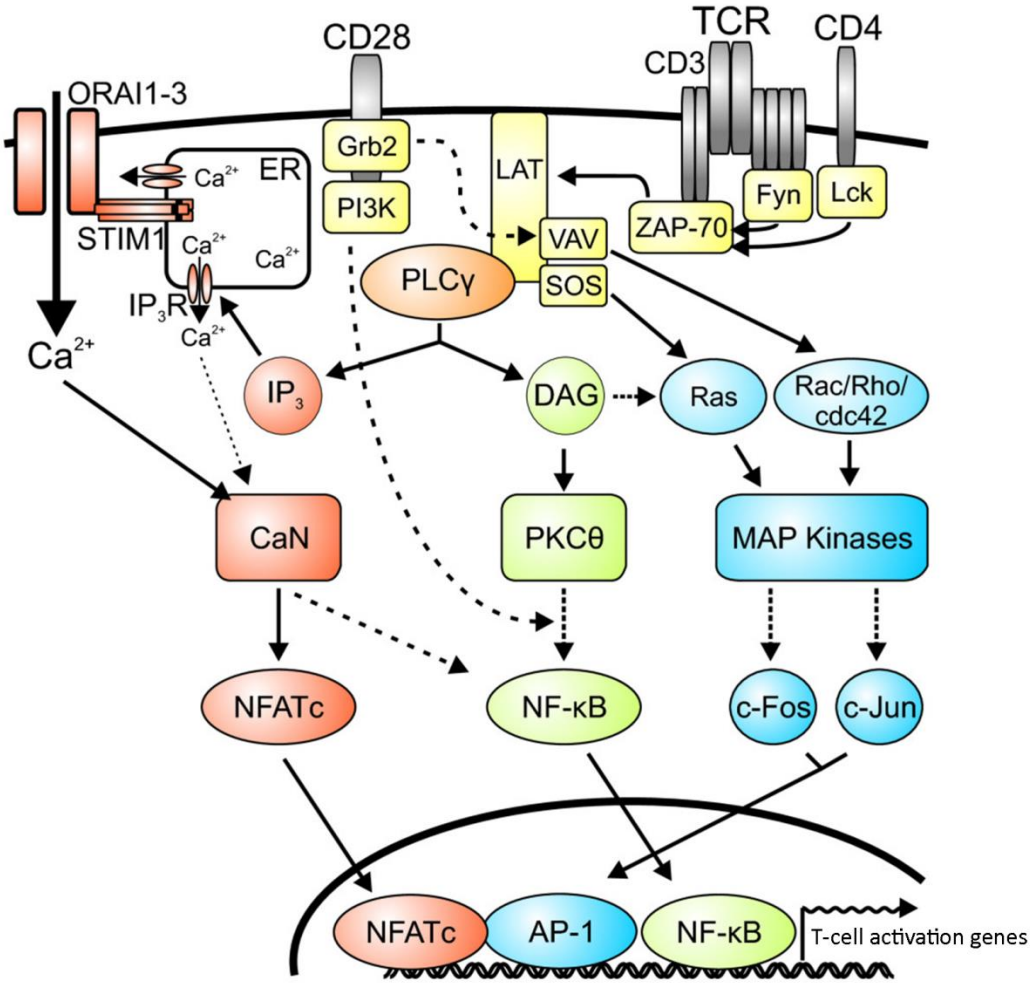


Figure 1.5. T-cell activation.

TCRs, in combination with CD3 molecules, drive signalling through the T-cell to activate numerous downstream signalling pathways and calcium influx, leading to changes in gene expression (adapted from [53]).

1.2 CD4+ T-cells and cancer

This thesis explores the potential of MHC class II restricted TCRs for the treatment of cancer. Whereas this T-cell subset was initially thought to only provide helper functions to other immune cells, there is now ample evidence that these cells are also capable of direct cytotoxic responses towards their targets, which include tumour antigens. Here I will consider these functions in relation to cancer immunology.

1.2.1 Immune surveillance and defence against cancer

The interplay between the immune system and cancer has been summarised in the immunoediting model. This model takes into consideration the paradoxical effects that the immune system has on tumour development. The immune response both sculpts tumour development by pressuring tumour cells to be less immunogenic, and prevents tumour development by killing tumour cells [54]. The three processes of immunoediting are described with three E's: elimination, equilibrium and escape.

Elimination is the immune mediated destruction of tumour cells. Both innate and adaptive arms of the immune system work in synergy to destroy transformed cells which are recognised due to tumour associated antigens being displayed on the cell surface. Pivotal work in this field was performed after the generation of Recombination Activating Gene (RAG) knock-out mice. As the RAG gene is responsible for the activation of TCR and B-cell Receptor (BCR) recombination, these mice lack T- and B-cells [55]. RAG-/- mice injected with chemical carcinogens form tumours faster that their wild type controls. This suggests that in the wild type mice, lymphoid cells are controlling tumour outgrowth, with the absence of clinical symptoms [56]. Spontaneously developed tumours are similarly eliminated by wild

type mice more efficiently than RAG-/- mice. If elimination of the cancerous cell(s) isn't complete, the second stage of immune editing begins, and equilibrium is established between the immune system and the cancer.

In this stage, the immune system continues to attack the genetically unsTable pre-malignant cells, driving selection of immune-resistant cells. Immune resistance is achieved by either reducing immunogenicity (immune evasion) or suppressing immune responses (immune suppression). Immune evasion often involves the reduction of cell surface MHC class I or II, thereby limiting antigen presentation [57, 58]. Additionally, malignant cells can have deregulated antigen processing and presentation pathways, again, limiting the presentation of antigen to immune cells. Alternatively, the reduction or cessation of mutated protein synthesis will prevent mutated peptides being presented to the immune system (epitope loss) [59]. Malignant cells have a multitude of mechanisms to suppress the immune response, and indeed the microenvironment is also often immunosuppressive. Malignant cells can express inhibitory molecules such as PDL1, and secrete molecules such as kynurenines, which can induce Th1 T-cell apoptosis [60, 61]. This selection of immune resistant cells highlights the dual effects of the immune response; it is able to both eliminate cancerous cells, yet shape them to avoid detection and develop into malignant disease.

Finally, if transformed cells are able to successfully evade the immune response, they will escape, proliferate uncontrollably and form tumours.

1.2.2 CD4+ Helper functions in cancer

Many groups have demonstrated that CD4+ T-cells are capable of orchestrating anti-tumour immunity through the production of a range of cytokines (reviewed in [62]). These cytokines are T-cell lineage dependent and have pleotropic effects within the immune response. Important for anti-tumour immunity are the cytokines produced by the CD4+ Th1 T-cell subtype, namely IFNγ, Tumour Necrosis Factor alpha (TNFα) and IL2.

One such example of immune modulation by cytokine production is the induction of tumour specific cytotoxic T-lymphocyte (CTL) responses, shown in mice by Nishimura et al. [63]. Here they show that Th1 T-cells are effective at eradicating tumours by stimulating CD8+ T-cells through the production of IFNγ. Others have identified the ability of tumour-specific CD4+ T-cells to aid CTL recruitment, survival and proliferation; responses driven by specific chemokines and cytokines [64].

CD4+ T-cells have also been shown to indirectly stimulate CTLs via conditioning or "licensing" of DCs [65-68]. Activated CD4+ T-cells express CD40L, which binds to CD40 on immature DCs to drive their maturation [69]. Mature DCs are better equipped to activate CD8+ T-cells, as they have upregulated surface expression of CD80 and CD86 [70].

Another example of immune modulation by CD4+ T-cells has been described by Perez-Diez et al. The group investigated monoclonal tumour-specific CD4+ T-cell responses *in vivo*. Results show that these cells were more efficient at clearing tumours than their CD8+ counterparts. As this effect is retained in *MHC class II* negative tumours, they suggest that here the CD4+ T-cells could function indirectly by maturing DCs and recruiting macrophages and natural killer (NK) cells [71].

Further evidence for the anti-tumour response of CD4+ T-cells is highlighted in mouse models and human trials. For example, Muranski et al. have shown that Th17+ T-cells are capable of eradicating established melanoma in mouse models – a result that was dependent upon the production of IFNγ [72]. Interestingly, Frankel et al. have described CD4+ T-cells as being as effective at tumour clearance as CD8+ T-cells, when transduced with the same, HLA-A2-restricted, CD4/CD8 independent, TCR [73]. As they have a reduced ability to lyse tumour cells when compared to tumour specific CD8+ T-cells, they suggest that the indirect helper effects contribute to the tumour control [72, 73].

1.2.3 CD4+ Effector Functions

In addition to this classic, indirect CD4+ T-cell response, recent studies have highlighted the direct effects that CD4+ T-cells can have on tumour clearance. This direct cytotoxicity has previously been attributed to an artefact of *in vitro* culture. However, studies investigating CD4+ T-cell cytotoxic responses *ex vivo* have validated this effector function [74, 75].

In a mouse model designed by Xie et al., MHC class II restricted transgenic T-cells specific for a melanoma specific antigen, tyrosinase, were adoptively transferred into tumour bearing mice. Regression was noted independently of vaccination, NK, NK-T, B or CD8+ T-cells. As Fas Ligand (FasL) and granzyme B were upregulated in the transgenic T-cells, the mechanism of killing was suggested to be through this pathway [76].

Human CD4+ T-cell anti-tumour responses have also been studied closely over the past decade.

Firstly, an increase in the frequency of tumour infiltrating CD4+ T-cells in patients with Diffuse Large B-cell Lymphoma (DLBCL) has been positively correlated with an improved

prognosis, highlighting the importance of this T-cell subset in anti-tumour immunity [77]. Additionally, CD4+ T-cells have been successfully isolated from the peripheral blood of patients with B-cell Chronic Lymphocytic Leukaemia (CLL) and shown to be cytotoxic towards CLL cells *in vitro [78]*.

Adoptive T-cell therapy is further highlighting the importance of CD4+ T-cells in tumour clearance. Dudley et al. have identified the CD4+ T-cell influence in tumour infiltrating lymphocyte (TIL) transfer, as patients receiving both CD4+ and CD8+ T-cells showed greater tumour responses overall [79]. Consequently, a number of groups have looked at transfusion of tumour reactive CD4+ T-cells alone. Both Hunder et al. and Tran et al. have demonstrated that in case studies, CD4+ TIL therapy is effective in the treatment of metastatic melanoma and metastatic cholangiocarcinoma, respectively [80, 81].

1.3 Immunotherapy overview

It is clear that the immune system can target cancer, and also that the immune system does not always do so effectively, as cancer is common, worldwide disease. How to best harness anti-tumour immune responses for cancer therapy is a question that is currently being investigated by researchers worldwide.

There is now a huge focus on immunotherapy. Immunotherapy is a term which describes the generation, activation or enhancement of a desired immune response in order to treat disease. There are multiple forms of immunotherapy including vaccines, immune checkpoint blockade inhibition and adoptive therapy.

1.3.1 Vaccines

Vaccines enhance the immune response towards specified antigens. There are many different types of vaccines currently being used clinically. Vaccines can include whole proteins, peptides, RNA or DNA. Clinical success with vaccines has to date been seen when vaccines are used prophylactically. Remarkably, by prophylactically treating smallpox we have managed to eradicate this disease from the world [82]. In terms of vaccinating against specific cancers, many challenges have hindered success in this field, including tumour driven immunosuppression and immune evasion. There are now prophylactic vaccines for the prevention of Human Papilloma Virus (HPV) associated cervical cancer. Prophylactic vaccines against other cancers such as breast cancer are currently under development [83]. Therapeutic cancer vaccines are currently being developed. For example, an EBV vaccine for the treatment of EBV associated malignancies and vaccines to treat glioblastoma are now in clinical trials [84-86].

1.3.2 Checkpoint Blockade Inhibitors

Checkpoint blockade inhibitors activate immune cells which may otherwise have been inactive, by removing the ‘brake’. When immune cells (specifically, T-cells), interact with their target they are activated to respond. Part of this activation includes a negative feedback loop, resulting in the expression of inhibitory signalling molecules such as CTLA-4, which leads to T-cell anergy, preventing T-cell proliferation and response [87]. CTLA-4 can be blocked by the monoclonal antibody Ipilimumab. This has shown clinical successes in the treatment of metastatic melanoma, increasing patient survival for 4 months when compared to a vaccine [88]. Whilst this effect may seem limited, it is the first treatment to ever show an

improved patient survival in a randomised trial for metastatic melanoma [88]. Ipilimumab is currently being tested for the treatment of other tumours such as non small cell lung cancer and metastatic prostate cancer [89, 90].

The Programmed Cell Death 1 (PD1) – Programmed Cell Death Ligand 1 (PD-L1) signalling pathway is another pathway which will inhibit T-cell responses; proliferation, survival and effector functions are all impaired after PD1-PD-L1 signalling [91]. PD1 is a receptor that is expressed on activated T-cells and often, tumours will express its ligand, PD-L1 [91]. Interaction of the receptor and its ligand can be blocked by antibodies against either molecule. These have shown to have great anti-tumour effects clinically, with anti-PD1 or anti-PD-L1 antibodies being approved for an increasing number of tumours every year [92].

1.3.3 Adoptive Therapy

Adoptive therapy is an arm of immunotherapy which refers specifically to the transfer of antigen specific immune cells (autologous or non autologous) into a patient, in order to achieve a desired response. This can activate and enhance responses by culturing immune cells in the absence of the immunosuppressive microenvironment. Additionally, adoptive therapy can generate novel immune responses, by genetically engineering immune cells to redirect their specificity.

1.3.4 Donor Lymphocyte Infusion

Donor Lymphocyte Infusion (DLI) involves the transfer of T-cells from a donor to a recipient. This occurs following HSCT which is used to treat cancers of the hematopoietic system. Prior to transplant the patient's immune system is ablated using total body irradiation and

chemotherapeutic agents such as cyclophosphamide to remove cancer cells [93]. Reconstitution of the donor derived immune system in these patients is slow. Neutrophils recover after 14-30 days depending on the type of HSCT (peripheral blood, bone marrow or cord blood derived stem cells). T- and NK-cells recover fully after 100 days and B-cells can take up to two years to recover [94]. Therefore, immune control of cancer relapses is limited. Here, DLIs are administered to increase the number of lymphocytes that will attack the tumour. DLIs are efficient at clearing recipient derived tumours as they are seen as 'non-self' and so destroyed [95]. The caveat to this therapy is that graft versus host disease (GvHD) is common.

1.3.5 Tumour Infiltrating Lymphocytes

Adoptive therapy with tumour infiltrating lymphocytes (TILs) requires isolation of TILs from a tumour biopsy, *in vitro* expansion and re-infusion into the patient. This therapy is autologous and thus removes the risk of GvHD. It can generate a range of T-cell specificities, reducing the risk of tumour escape by epitope loss. Unfortunately, the long expansion period of around 4-16 weeks is a major drawback in this therapy because cells can become exhausted with such expansions and patients treated by such therapy (most often metastatic melanoma patients) require rapid treatment; however shorter protocols are being developed. [79, 96]. TILs are found primarily in highly immunogenic tumours such as melanoma and thus isolation from less immunogenic tumours will prove difficult [97]. Additionally, TILs can only be generated if it is possible to biopsy the tumour. Despite these drawbacks, the therapy has seen clinical success in the setting of melanoma. Rosenberg et al. have reported a 72% object response rate when total body irradiation was used as a preconditioning regimen and complete remission was observed in 22% (20/93) of patients [98].

1.3.6 Cytotoxic T-Lymphocyte Infusion

Like TIL therapy, cytotoxic T-lymphocyte (CTL) infusion also requires the expansion of tumour specific T-cells. Here, T-cells are isolated from patient peripheral blood mononuclear cells (PBMCs), and target-specific T-cells are selectively expanded and infused into the patient. Alternatively, in HSCT, donor CTLs can be isolated and expanded. In one study treating melanoma patients, partial responses were reported in 8 of 20 patients treated [99]. Additionally, CTL infusion has been used to treat Hodgkin and non Hodgkin lymphoma, with responses seen in 13 of 21 relapsing patients, of which 11 responses were complete [100]. These trials did not see GvHD – a toxicity considered to be reduced in comparison to DLI due to the selective expansion of target specific T-cells, however GvHD will remain a safety concern when administering donor derived T-cells into immunocompromised patients [101].

1.4 Genetic Engineering of T-cells

It is not possible to isolate and expand naturally occurring tumour reactive T-cells from every patient, due to their low frequencies or absence. Genetic engineering of T-cells to redirect their specificity has been shown to be clinically effective and would allow for the treatment of patients who do not possess tumour reactive T-cells. Additionally, genetic engineering of T-cells bypasses the need for long T-cell expansions; it allows for the rapid production of many specific T-cells. Finally, these T-cells can be patient derived. The use of autologous T-cells reduces the complications associated with allogeneic T-cells, including rejection of the adoptively transferred cells and GvHD.

1.4.1 Chimeric Antigen Receptors

The first of these genetic engineering approaches is the use of chimeric antigen receptors (CARs). CARs are generated from the antigen specific fragment of an antibody (single chain variable fragment, ScFv), linked to a constructed signalling domain. T-cells transduced with this construct express it on their surface and bind to their cognate antigen with the high affinity of an antibody. What's more, CARs are not MHC restricted, and so any patient expressing the target antigen on their tumour cells could potentially be treated. Upon antigen binding, signalling through the CAR activates a T-cell response. To date, there have been numerous generations of CARs (Figure 1.6), which include various signalling domains from different co-stimulatory molecules [102-104] [105-107]. It is not yet known which combination of co-stimulatory molecules is optimal due to a lack of direct comparisons, and it is likely to change depending on the ligand being targeted.

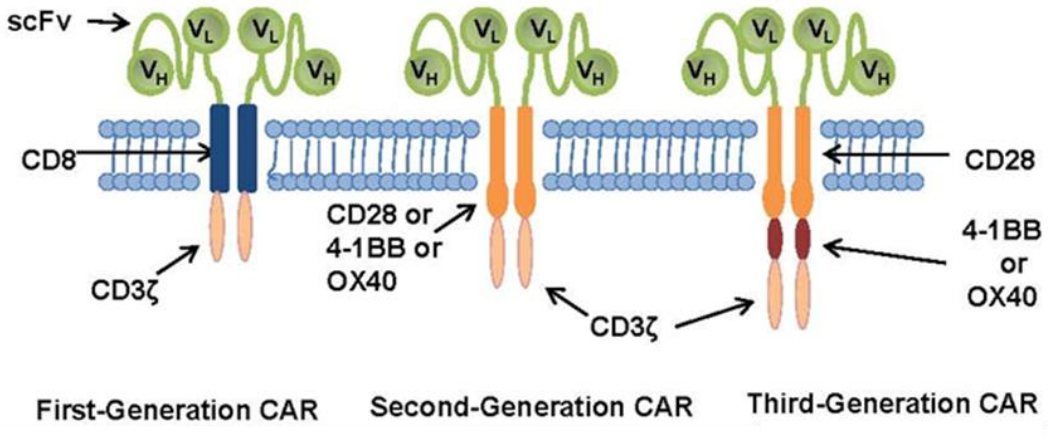


Figure 1.6. Generations of CAR constructs.

Three generations of CARs have been developed which include a range of signalling molecules. First generation CARs typically signal though CD3ζ whilst second and third generation CARs incorporate additional signalling domains (adapted from [108]).

Clinical trials that target Acute Lymphoblastic Leukaemia (ALL) and Chronic Lymphocytic Leukaemia (CLL) with second generation CD19 specific CARs have proven remarkably effective. Carl June's group have shown that these transduced cells are predominantly safe, persist, and have impressive anti-tumour effects [109]. Indeed, in a trial treating 30 ALL patients, 90% of patients entered complete remission [110]. However, the strength of this therapy has also been witnessed in adverse effects and patient mortality using CARs directed towards other targets. When T-cells have been administered to patients with large tumours, copious amounts of cytokines have been produced during the rapid tumour lysis. This is known as a cytokine storm and can be toxic [111]. Furthermore, CAR transduced T-cells specific for targets that are present at low levels in normal tissues yet upregulated in cancer have attacked the normal tissues. In rare cases this on target off tumour toxicities has resulted in the death of the patient [109, 112].

1.5 T-cell Receptor Gene Transfer

Following initial successes of adoptive cell transfer with TILs and CTLs, there has been much interest in using TCR gene transfer as a therapeutic approach to target tumours. TCR gene transfer is a method of genetically engineering T-cells to bestow upon them a specific reactivity. T-cells with defined specificities are isolated, and the genes which encode their TCRs are cloned into retroviral or lentiviral constructs. These constructs are subsequently transduced into activated T-cells, which will then express the specific TCR (Figure 1.7). Unlike CARs, TCRs are HLA-restricted. Consequently the patient can only be treated if they express the HLA allele that the transduced TCR is restricted by. However, TCRs have a wider peptide repertoire than CARs since unlike CARs they can recognise not only surface

antigens, but antigens derived from any compartment of the target T-cell, although this is dependent on a functioning MHC processing pathway.

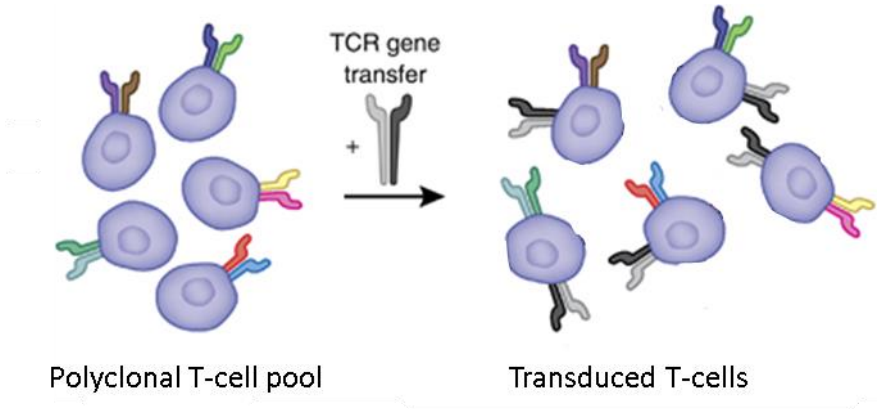


Figure 1.7. TCR gene transfer.

Polyclonal T-cells are redirected towards new antigen specificity by TCR gene transfer (adapted from [113]).

1.5.1 History of TCR gene transfer

The first demonstration of redirecting T-cell specificity was seen in 1986, when Dembic et al. transferred TCR genes from one CTL to another, successfully redirecting it's specificity [114]. Since then, there have been numerous clinical trials and much preclinical research into TCR gene transfer.

Using a TCR specific for a HLA A2 restricted melanin antigen, Clay et al. acknowledged the redirection and anti tumour activity of TCR transduced cells *in vitro* [115]. Kessel et al. subsequently demonstrated that redirected T-cells can function *in vivo* [116] . Work within this field then bloomed, with multiple clinical trials currently on-going. The first clinical trial

for TCR transfer involved the transfer of a TCR specific for a Melan-A (MART1) antigen (DMF4) for patients with melanoma. Early results showed an overall response rate in 2/15 (13%) of patients [117]. Subsequent trials with higher affinity TCRs and improved transduction efficiencies have resulted in improved responses. By using the DMF5 MART1 specific TCR, which has a 100 fold increase in avidity compared to DMF4, a 30% response rate was observed (6/20 melanoma patients) [118]. The disadvantage of this TCR is its on target side effects, as non-tumour cells which express this antigen at low levels were destroyed, resulting in toxicities. These on target side effects highlight the potential of TCR transfer as an effective cancer therapy, providing conditions are optimised to increase efficiency and reduce toxicities.

1.6 TCR Gene Transfer Optimisation

1.6.1 Mispairing

When exogenous TCR alpha and beta chains are introduced into T-cells, they are theoretically able to pair with endogenous TCR chains. This mispairing results in TCRs with unknown specificity – a risk that has been shown to be associated with lethal GvHD *in vivo*, at least in mice [119]. Additionally, mispairing TCR chains will reduce the amount of fully formed exogenous TCRs at the cell surface, thereby reducing T-cell sensitivity. Due to these disadvantages, a number of groups have developed ways to reduce this mispairing.

Creating an additional disulphide bond between the alpha and beta TCR chains has been shown to aid pairing between exogenous TCR chains, thereby increasing the surface expression of the exogenous TCR [120].

Another method used to improve TCR chain pairing is to use mouse constant domains, with the V, D and J domains remaining human [121, 122]. By using this technique, surface expression of exogenous TCR can be increased by more than two fold [122].Due to concerns that this technique would result in loss of TCR-transduced cells in the host, as human anti-mouse immune responses develop, some groups choose to utilise minimal murine constant domains. These would retain TCR pairing yet be less likely to stimulate an anti-mouse response [123].

Recent work has focused on preventing TCR mispairing by removing the endogenous TCR altogether. This not only increases the safety profile of the therapy, but also improves TCR expression at the cell surface, as the introduced TCR will no longer have to compete with the endogenous TCR for CD3 binding, which is required for surface expression [124]. There are multiple methods in place to remove the endogenous TCR. Zinc finger domains have been utilised to disrupt the expression of endogenous alpha and beta chains [125]. Whilst this method is effective, it considerably increases the amount of time the T-cells are *in vitro*, the complexity of the protocol and the cost, which is a clear disadvantage for this therapy. RNA interference molecules (RNAi) have been used to remove the endogenous TCR [126]. RNAi silences transcription of the endogenous TCR genes and results in equal surface expression of the exogenous alpha and beta chain, suggesting improved pairing [126.]. Finally, others have transduced hematopoietic stem cells (HSCs), which do not express endogenous TCRs. This technique has shown to generate functional T-cells [127]. However, the added complexity of obtaining these cells complicates this protocol for widespread clinical use.

Although TCR mispairing remains a safety concern, no lethal toxicities due to mispairing have been identified clinically to date in the human setting [128].

1.6.2 Increasing surface TCR

Along with ensuring correct pairing, techniques are commonly employed to increase surface expression of the TCR. Firstly, codon optimisation is frequently used to improve translation of TCR messenger ribonucleic acid (mRNA). Codon optimisation is the process of changing nucleotides by site directed mutagenesis to other nucleotides without altering the resulting amino acid sequence, in order to improve translation efficacy. This is possible because triplet codons are degenerate; multiple triplet codons can result in the incorporation of the same amino acid. Some triplet codons are more easily translated by ribosomes and so if the DNA sequence is altered to include the better translated triplet codons, translation as a whole is more efficient. This method was originally used in TCR gene transfer by Scholten et al. and resulted in substantially increased surface expression of the exogenous TCR [129].

TCRs must bind to CD3 for surface expression. The competition for CD3 can be removed either through deleting the endogenous TCR as described above, or by the addition of more CD3. Ahmadi et al. have investigated the co-transduction of CD3 and TCR genes, and show that this enhances both TCR surface expression and T-cell function [130].

1.6.3 Transduction vehicle

To date most of the preclinical and clinical studies of TCR gene transfers use a retrovirus or a lentivirus system to transduce TCR genes into cells.

Retroviruses integrate non-specifically into the host cell genome, often close to transcription start sites [131]. Although this could theoretically result in integration next to oncogenes and drive tumour development, this has not been seen clinically, and patients who were treated

with retroviral constructs over 12 years ago have not developed retroviral driven tumours [132, 133]. Lentiviruses are considered 'safer' than retrovirus as they have a more selective integration site, and will often integrate within active transcription units [134]. However the transposon/transposase system may be used in the future, and prove to be safer. Transposon/transposase systems have been shown to only integrate into a few sites in the genome and are unlikely to integrate into transcriptionally active genes [135, 136]. The SB system has been developed for clinical use and tested in a phase I/II clinical trial to transduce a CD19 CAR into T-cells [137]. The SB system likely requires further optimisation before it will be used commonly within the field, as currently transduction is inefficient. In a second CD19 CAR trial which used SB to transduce cord blood cells, 4 cycles of electroporation of the transposon and transposase was typically required to generate enough transduced T-cells for clinical use, which took 28 days [138].

1.6.4 Clinical Results

There have been numerous TCR gene transfer therapies in the clinic, with a range of epitopes being targeted. To date, these therapies have been studied in most depth in metastatic melanoma and lymphomas. Synovial cell carcinoma, myeloma and chronic lymphocytic leukaemia have also been treated clinically [117, 139-144].

In general terms, responses are varied and toxicities are common, highlighting the requirement to identify tumour specific antigens. The first clinical trial in 2006 showed modest tumour regression, with 2/15 patients with metastatic melanoma achieving an objective response [117]. Since this time, TCR gene transfer has been optimised (as discussed above), and now we are seeing trials with greater responses. A trial by Robbins et al. in 2011 achieved responses in 4/6 patients with synovial cell carcinoma and 5/11 patients with

melanoma. However, as TCR gene transfer is being optimised and thus becoming more potent, more toxicity is occurring, highlighting the strength of this therapy. In 2015, van den Berg et al. reported a mortality in a patient treated with a MHC class I restricted TCR specific for MART1 [112]. Toxicities were determined to be due to cytokine release syndrome, highlighting the potency of this TCR. Additionally, in 2013, a trial was performed to treat patients with myeloma and melanoma with an affinity enhanced TCR targeting MAGE A3. Following infusion of these affinity enhanced TCR transduced T-cells into two patients, the patients developed cardiogenic shock which led to their death and termination of the trial [144]. In depth investigation revealed that the TCR was cross reactive to a protein (titin) which is present on beating cardiomyocytes. This cross reactivity was not noticed prior to the trial as titin is not expressed on cardiomyocytes *in vitro* unless grown as beating cardiomyocyte cultures. This trial highlights the requirement for safety measures within this therapy, and many methods to increase safety have been developed, including the use of suicide genes to provide control over harmful T-cell responses [145].

1.6.5 Suicide Genes

The administration of T-cells for therapeutic intervention differs from the administration of molecular drugs as T-cells are able to expand *in vivo* and persist for years [132, 133]. These reasons are what make adoptive T-cell therapy of cancer so exciting; one cell product could expand or decrease depending on the target availability and immune memory can be generated for the prevention of tumour relapse. On the other hand, expansion and persistence of infused T-cells could be lethal if the T-cells administered have unexpected toxicities. As shown in the pre- and post-clinical evaluation of the titin cross reactive TCR, the specificity of genetically engineered T-cells cannot always be completely defined *in vitro*. Regardless of

the *in vitro* safety profile of the TCRs, suicide genes could be transduced into the T-cells to give clinicians some control over toxicities. Suicide genes can be activated to kill the transduced T-cells if unwanted toxicities are witnessed. Examples of such suicide genes include the Herpes Simplex Virus Thymidine Kinase (HSV TK) gene and the inducible caspase-9 safety switch. The HSV TK phosphorylates the nucleoside analogue ganciclovir. The phosphorylated from of ganciclovir is incorporated into DNA and prevents DNA replication, ultimately resulting in cell death [146]. This suicide gene is consequently inducible; it only functions after the administration of ganciclovir. HSV TK has been used in clinical trials and shown to be safe, however when transduced into T-cells for DLI, not all T-cells were killed and thus its efficacy is limited [147, 148]. The caspase-9 safety switch is made of a human caspase-9 linked to the human FK506 binding protein (FKBP). This is transduced into T-cells. A synthetic molecule which induces dimerisation of the caspase-9 molecules can be administered if ablation of genetically engineered T-cells is required. Dimerised caspase-9 is active and causes a caspase cascade and consequential apoptosis of 99% of the transduced cells [149]. This safety switch has been tested in a clinical trial where it was transduced into donor T-cells prior to HSCT. When a patient developed GvHD the small molecule was administered. This resulted in elimination of 85-95% of infused T-cells within 30 minutes and rapid resolution of GvHD [150]. As well as being very efficient, this caspase-9 safety switch has other benefits over the HSV TK gene transduction. As it is made of human genes, T-cells transduced with it will not be targeted for destruction by the host's immune system (as has been shown in the HSV TK system) [151]. Secondly, the pro-drug is non-toxic, unlike ganciclovir, which is an antiviral used for the treatment of CMV infections and is associated neutropenia [152].

First Author	Target Tumour	Target Epitope	MHC	T-cell Subtype	Patients	Results	Toxicities	Other Notes
Morgan (2006) [98]	Melanoma	MART-1	I	Total PBMC	15	2 objective regressions	No toxicities attributed to engineered T-cells	TIL derived TCR
Johnson (2009) [117]	melanoma	MART-1	I	Total PBMC	20 (DMF5) 16 (GP100)	30% (DMF5) and 19% (GP100) objective response	Vitiligo and melanocytes destruction in majority of patients. Hearing loss	DMF5 TCR from TIL GP100 from HLA Tg mice
Parkhurst (2011) [118]	Metastatic colorectal cancer	CEA	I		3	1 objective response	Severe transient inflammatory colitis	Murine TCR (tg mouse)

First Author	Target Tumour	Target Epitope	MHC	T-cell Subtype	Patients	Results	Toxicities	Other Notes
Robbins (2011) [119]	Metastatic Melanoma Synovial Cell Carcinoma	NY-ESO1	I	Total PBMC	SCC – 6 Melanoma - 11	SCC - 4 responders Melanoma – 5 responders	Transient neutropenia and thrombocytopenia in response to lymphodepleting regimen and high dose IL2 infusion	Patient derived TCR, Two amino acid substitutions in CDR3
Seaman (2012) [120]	Progressive Metastatic Melanoma	MART-1 GP100	I		MART-1 - 18 GP100 - 14		25% transient hearing loss due to autoimmune attack of melanocytes	MART-1 from Tg mice, GP100 from TIL

First Author	Target Tumour	Target Epitope	MHC	T-cell Subtype	Patients	Results	Toxicities	Other Notes
Morgan (2013) [121]	Multiple epithelial tumours	MAGE-A3	I	Total PBMC	9	5 patients showed clinical regression	3 mental status changes, 2 deaths - cross reacted to mage A12 found in brain	HLA transgenic mouse and site directed mutagenesis
Linette (2013) [122]	Myeloma and Melanoma	MAGE-A3	I	CD25 and monocyte depleted PBMC	2	N/A	Both patients died from myocardial infarction and myonecrosis Recognistion of unrelated peptide	Affinity enhanced TCR

Table 1.1 Table of TCR Gene Transfer Clinical Trials to date.

1.7 Epstein Barr Virus

EBV is a 172 Kb double stranded linear DNA gamma herpes virus that infects around 90% of the world's population [153]. This genetically sTable virus is usually transmitted by salivary exchange. Infection generally occurs asymptomatically in childhood, although in the western world it is often delayed until adolescence. Infection results in Infectious Mononucleosis (IM) in up to 77% of adolescents, however this frequency is much lower when children are infected [154, 155]. The acute infection is eventually brought under control by the immune system [153]. The virus enters latency and persists as a lifelong infection.

1.7.1 Life Cycle and Gene Expression

EBV naturally infects B cells that are transitioning through the oropharyngeal epithelium. Through the viral glycoproteins gp350 and gp42, EBV binds to the chemokine receptor CD21, and MHC class II respectively, on the surface of B-cells. This is thought to happen in the oropharynx during viral infection of the host [156-158]. From here, the virus can infect epithelial cells in the oropharynx and replicate, causing cell lysis and virion release [159]. The released virions can infect naive B-cells by CD21 mediated endocytosis [160]. Upon infection the virus adopts a largely latent state with the expression of nine growth transforming "latent" viral proteins (these proteins are listed in Table 1.1), in a state known as latency III [161]. The rich array of immunogenic lytic and latent antigens present during acute infection drives a strong T-cell response which eventually brings the primary infection under control. However, some infected cells escape elimination and viral gene expression is completely shut down (latency 0) [162]. These resting memory B-cells circulate the blood indefinitely, avoiding immune recognition [3]. They undergo periodic lytic reactivation when circulating through

the oropharynx to produce new virus progeny for transmission and infection of new B cells [163]. EBV DNA is not integrated into the cell DNA; it is maintained in an episomal manner [164]. Host cell DNA polymerases replicate EBV DNA, which is spread equally into daughter cells before B-cells divide [165].

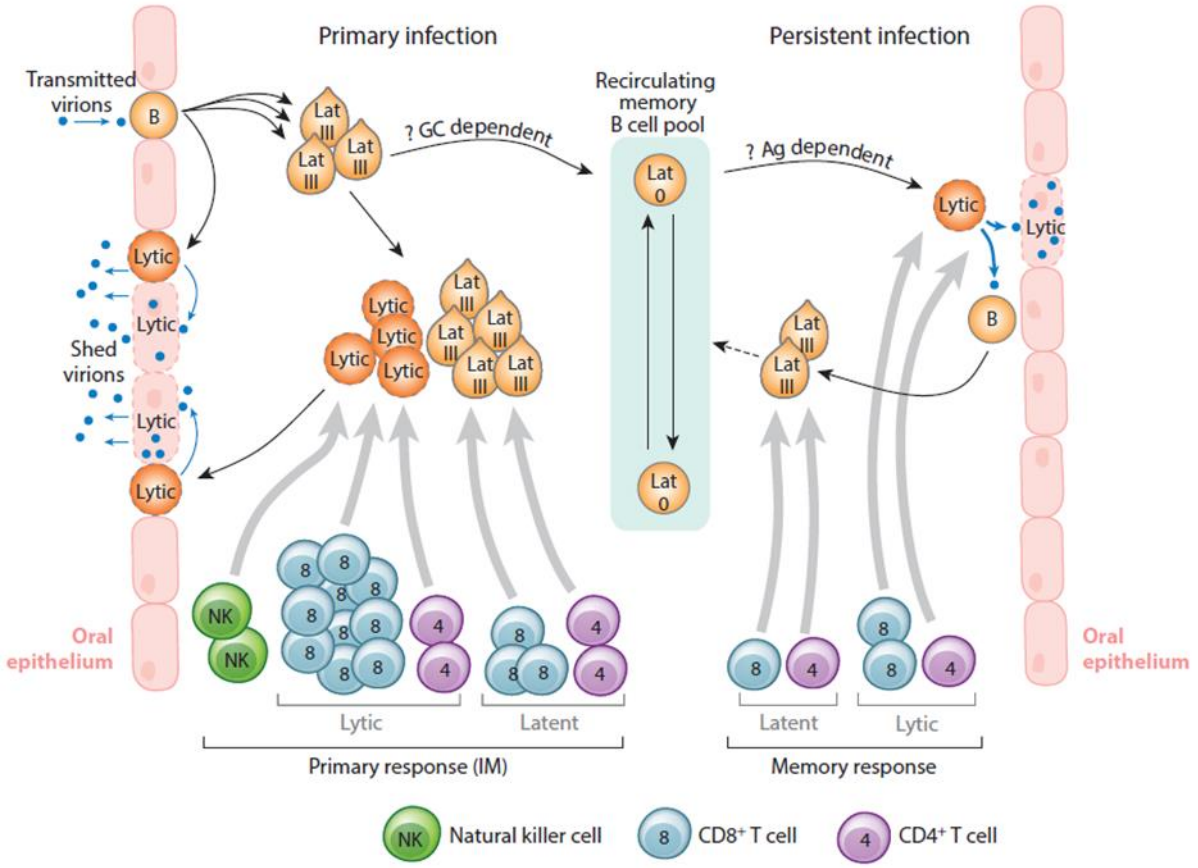


Figure 1.8 EBV life cycle and immune response.

EBV primarily infects epithelial cells. Here EBV replicates to produce new virions for oral transmission and infection of B-cells. EBV infected B-cells switch to latency 0 in response to NK, CD4+ and CD8+ T-cell pressure. EBV lytic gene expression occurs in infected memory B-cells to allow for virus transmission. Taken from [166].

The EBV lytic replicative cycle produces new virus particles for the spread of infection. Lytic cycle leads to cell lysis and thus the release of newly made EBV virions, allowing for the infection of more naive B-cells [167]. Expression of lytic genes is driven by the immediate

early (IE) lytic protein, the transcription factor BZLF1. Lytic gene expression is staggered into immediate early, early and late gene expression. In these stages, different lytic genes are expressed, with the total number of lytic genes exceeding 80. A constant low level of viral shedding in the throat of healthy carriers suggests that a small proportion of the virus enters lytic cycle at any given time, probably due to antigenic stimulation of B-cells (Figure 1.8) [153].

Programme	Tumour association	Gene expression
Latency III	PTLD AIDs related lymphoma DLBCL	EBNA-LP, EBNA1, EBNA2, EBNA3a, EBNA3b, EBNA3C, LMP1, LMP2A, LMP2B, BHRF1, BARTs, EBERs.
Latency II	Hodgkin's Lymphoma DLBCL NPC T/NK cell lymphoma Gastric Carcinoma	EBNA1, LMP1, LMP2, EBERs
Latency I	Burkitt's Lymphoma T/NK cell lymphoma Gastric Carcinoma	EBERs and EBNA1

Table 1.2 EBV gene expression profiles in tumours.

EBV is associated with the tumours described in this Table and expressed most commonly a latency I, II or III gene programme.

1.7.2 Gene Function

EBV has growth transforming potential through the coordinated actions of the latent proteins, which can be seen *in vitro* as the outgrowth of latency III positive lymphoblastoid cell lines (LCLs) upon infection of B cells with EBV. However, some tumours display more restricted patterns of EBV gene expression (Table 1.1). Here, cellular changes in tumours with more complex aetiologies can compensate for some transforming functions.

EBV nuclear antigen (EBNA) 1 is a nuclear protein which binds to both viral and host DNA during mitosis to ensure the viral episomes get integrated into the daughter B-cells [168]. It has been proposed to prevent apoptosis in BL [169]. EBNA1 also acts as a transcription factor, negatively regulating itself and positively regulating LMP1 and Cp promoters [168, 170]. EBNA2 is a transcription factor which leads to transcription of all latent genes and thus has a transforming role. This transforming role can be shown by rescuing the transformation ability of EBNA2 mutants with wild type EBNA2 [171]. It can bind to c-myc to activate it and drive cell proliferation and additionally has partial homology to NOTCH, allowing it to block cell differentiation and so maintain a proliferating state [171]. EBNA3 proteins are transcriptional regulators for viral and host genes [172]. Both EBNA3a and 3c are essential for *in vitro* transformation [173]. EBNA-LP can bind to pRb and p53 although the functional significance of these interactions is so far unclear [174]. However it is required for LCL outgrowth and has been proposed to be a transcriptional activator [168]. LMP1 is a major transforming protein, indispensable for B-cell transformation [171]. It acts as a ligand independent, constitutively active CD40 homologue and signals via cell signalling molecules to activate MAPK, NFkB and PI3K pathways[175]. In this manner it increases expression of cytokines and antiapoptotic proteins, aiding cell growth and survival [176]. LMP2 aids B-cell

survival by signalling in a similar manner to BCR signalling, activating both PI3K and MAPK pathways [171]. By sequestering the binding proteins required for BCR signalling it inhibits this pathway and so prevents B-cell differentiation, helping to maintain a latently infected B-cell pool [177]. EBV encoded RNAs (EBERs) are non-polyadenylated RNA structures which are suggested to inhibit RNA-dependent protein kinase (PKR) activation. PKR activation facilitates the anti-EBV effect of IFNs and so by inhibiting this kinase, the immune response to EBV is diminished, enhancing viral persistence [171, 178]. Finally, BamHI A rightward transcripts (BARTs) have no known function yet are expressed in healthy carriers and in tumours and therefore are thought to have a role in viral persistence [171]. As the tumorigenic properties of lytic genes are less clear, they will not be discussed here.

1.7.3 EBV tumour association

It is estimated that 200,000 cases of EBV-associated cancer occur annually [179]. EBV has been detected in multiple different B-cell tumours including; Hodgkin's Lymphoma (HL), Burkitt's lymphoma (BL), PTLD, DLBCL of the Elderly and AIDs-related lymphoma. These tumours express different EBV gene expression profiles, as can be seen in Table 1.1. EBV has further been identified in some epithelial cancers such as nasopharyngeal carcinoma and in T-cell lymphomas [171]. The role of EBV in tumourigenesis has been debated due to cases of apparently EBV negative HL and BL. However, tumours negative for EBER expression (a common marker used to identify EBV infection) can test positive for EBV DNA [180]. A 'hit and run' hypothesis has been suggested in which EBV was originally in the B-cell but was lost when it was no longer needed; e.g. when the cell had undergone enough genome damage to be tumorigenic [177].

1.7.4 Immune Response to EBV

Patients diagnosed with IM have proved invaluable for investigating the immune response to EBV. Peripheral blood samples taken from donors with IM have shown an increase in NK cell numbers, when compared to healthy donors [181]. The NK cells that proliferate in response to EBV infection are $CD56^{dim}$ and CD16-, which *in vitro* are most efficient at responding to lytically infected cells [181]. These NK cells persist for several months after infection [182]. *In vitro* studies of innate immune responses to EBV have shown that monocytes and DCs can also respond to this virus [183].

Adaptive immune cells also respond to EBV, and together with innate cells drive EBV latency 0, as infected cells which express EBV genes are cleared. B-cells respond to EBV infection by producing antibodies. IgG antibodies are initially detected following primary EBV infection against EBNA2 and these subsequently decline [166]. One way in which EBV serostatus is determined in healthy people is to test for the presence of IgG against VCA and EBNA1, as these are maintained in the B-cell pool following primary infection [166, 184].

Both CD4+ and CD8+ EBV specific T-cells proliferate in IM, with clinical symptoms coinciding with CD8+ T-cell expansion [154]. Indeed, in children where primary EBV infection does not result in IM, the total number of peripheral blood CD8+ T-cells are not significantly changed to those of healthy controls [185]. This suggests the clinical symptoms of IM are a result of CD8+ T-cell expansion. Huge numbers of CD8+ T-cells can be identified in IM patients, and up to 40% of peripheral blood CD8+ T-cells can be specific for single EBV derived lytic antigens [153]. Immunodominance is observed within the CD8+ T-cell response to EBV, with the majority of peripheral blood derived CD8+ T-cells being specific for lytic antigens. Responses against IE antigens are the most prevalent, followed by E and

then L. Responses against latent antigens are observed in the blood from IM patients, albeit at lower frequencies. Up to 5% of total peripheral CD8+ T-cells can be specific for latent antigens. As acute infection is cleared, the EBV specific CD8+ effector cells undergo apoptosis. IM is a self-limiting disease, with the protraction of EBV specific CD8+ T-cells correlating positively with reduced symptoms. The overall number of CD8+ T-cells eventually reaches that of a healthy EBV carrier. A small proportion of memory CD8+ T-cells specific for EBV remain to control persistent infection.

The investigation of the CD4+ T-cell response to primary EBV infection has met several challenges. These included the lower frequency of EBV specific CD4+ T-cells in the peripheral blood compared to that of the CD8+ T-cell counterpart, the lack of peptide-MHC multimers and the scarcity of knowledge of specific CD4+ T-cell specificities. Technological advancements – in particular the production of peptide-MHC multimers, has recently helped to overcome these challenges.

The CD4+ T-cell response to primary EBV infection differs to that of the CD8+ T-cells, as specific T-cell expansion is less pronounced [154, 186]. Proliferation of EBV specific CD4+ T-cells is observed in IM, with the most frequent T-cells recognising latent-derived antigens [187]. The response is spread throughout all latent genes (with the exception of EBNA1) and as such, no immunodominance is observed. T-cells specific to any one epitope can be found to constitute up to 1% of total CD4+ T-cells in the peripheral blood, with these frequencies after primary infection returning to levels seen in healthy EBV carriers [187]. The EBV specific memory CD4+ T-cell repertoire contains T-cells specific for antigens derived from latent and lytic proteins. This breadth of specificities is larger than that observed in memory CD8+ T-cells [153].

EBV specific CD4+ T-cells have been isolated from healthy EBV seropositive donors and shown to be able to produce multiple cytokines and also be directly cytotoxic towards EBV infected B-cells (LCLs) *in vitro*. Long et al. have described CD4+ T-cell clones specific for 12 latent antigens from 4 proteins, and 12 lytic antigens from 8 proteins, that are capable of producing cytokines and having direct effector roles [188, 189].

Direct effector CD4+ T-cell responses are possible here as the infected cell type expresses MHC class II. Endogenous viral antigens have been shown to enter the MHC class II processing pathway through multiple mechanisms. EBV proteins including EBNA2, EBNA3a, b and c enter the MHC class II processing pathway by intercellular antigen transfer to neighbouring cells, thus effectively acting as exogenous antigens [35, 190]. Antigens from these proteins are not thought to be processed via intracellular routes, as overexpression of antigen using vaccinia vectors and inhibiting autophagy do not increase CD4+ T-cell responses [35].

Other EBV proteins such as EBNA1 have been shown to enter the MHC class II processing pathway through intracellular routes. Intercellular routes have been dismissed through antigen transfer assays which co-culture antigen negative, HLA restricted recipient cells with antigen positive, HLA unrestricted donor cells. Some EBNA1 antigens have been proven to be presented through MHC class II as a consequence of autophagy [33, 191]. Interestingly, other EBNA1 epitopes that CD4+ T-cell clones can directly recognise enter MHC class II processing pathways by undetermined routes.

1.8 Post Transplant Lymphoproliferative Disease

The EBV associated malignancy we are particularly interested in here is PTLD. PTLD is a life threatening disease that occurs in roughly 1%-11% of patients following hematopoietic stem cell transplant (HSCT), and between 1 and 20% in solid organ transplants (SOTs) [192, 193]. 90% of PTLDs are EBV positive, as EBV is able to expand infected B-cells uncontrollably in the absence of immune pressure [194, 195]. The clinical symptoms of EBV-positive PTLD are diverse, and patients often present with symptoms similar to those of IM. PTLD is a heterogeneous disease. The World Health Organisation (WHO) has classified PTLD into four subgroups; early lesions, polymorphic, monomorphic, lymphomatous and Hodgkin lymphoma-like [196]. Cho et al. have characterised the EBV DNA in transplant patients and have identified that the peak EBV-DNA level is able to predict PTLD development [197]. Indeed, EBV load in patient blood is closely monitored post-transplant and patients are treated if this starts to rise [198].

1.8.1 Incidence and risk factors

A number of factors are associated with an increased risk of developing PTLD, accounting for the range in incidence. These include the type of organ transplanted, with lung, heart, liver and kidney transplants being associated with the lowest risk (1-10%) and the small bowel being associated with the highest risk (20%) [192].The amount of transferred lymphoid tissue is dependent upon the type of organ transplanted, and has been shown to influence PTLD [199] . The EBV sero-status of both transplant donor and recipient has a large influence in the development of PTLD, with EBV seronegative recipients and seropositive donors being at highest risk [200]. For this reason, there is a high incidence of PTLD within young children,

who are more likely to be EBV seronegative. Additionally, young children have been shown to be at higher risk of developing PTLD, regardless of prior EBV status, along with patients over the age of 60 [201, 202]. The type and intensity of immune conditioning prior to treatment also influences PTLD development, with more immune suppression leading to a greater risk of developing PTLD [202]. As SOT transplants remain on some level of immunosuppression throughout life, PTLD can develop at any point after transplant, and has been reported up to 14 years post-transplant [203]. Additional factors influence the risk of PTLD development following HSCT, with the greatest being the type of preconditioning. The incidence of PTLD increased with the introduction of T-cell depleted grafts, which was introduced in order to avoid GvHD [204]. This puts recipients at higher risk of developing PTLD of donor origin because of the lack of T-cells to control the infection. Finally, HLA mismatch can influence PTLD development. If the PTLD arises from the recipient and antigens are being expressed through HLA alleles that are not common between the donor and recipient, donor T-cells may not be able to control the lymphoproliferation completely. Together, these risk factors have a cumulative effect on the development of PTLD.

1.8.2 Clinical symptoms and morphology

As a heterogeneous disease, PTLD has many clinical and morphological manifestations. Depending on the time of PTLD onset, lesions may contain monomorphic or polymorphic cells. These cells may be monoclonal or polyclonal. Clinical symptoms are diverse, often including fever, sweats, malaise, weight loss, enlarged tonsils, cervical lymphadenopathy, organ dysfunction and infectious complications [192]. The median onset of PTLD is 6 months, yet it has been reported to arise from one week to over ten years after transplant [205].

Early-onset PTLD arises most commonly in HSCT patients, who are severely immunocompromised, within one year of receiving the transplant. These lesions are often polyclonal and polymorphic. Early-onset PTLD has a high association with EBV, with many cases expressing a latency III gene expression profile [206, 207]. Therefore, LCLs are a good model of PTLD. Indeed, they have been used extensively as a model of PTLD when investigating PTLD treatment options.

Patients who received SOT also most commonly develop PTLD within a year, as this is when immunodeficiency is greatest, although continuing immunosuppression means that PTLD may develop after a year (late-onset) [166]. In these conditions, EBV is able to transform B-cells. However, due to immune pressure (albeit limited compared to immunocompetent people) EBV gene expression is often restricted to avoid detection, and the tumours often carry cellular mutations such as p53, RAS or c-Myc. Hodgkin's like lymphoma PTLD expressing a latency II profile is common in PTLD following SOT. Lesions are often monoclonal and monomorphic. Further, EBV is less frequently associated with late-onset PTLD, with only 66% of cases being EBV positive [208]. One hypothesis for the development of EBV negative PTLD is that the lesions were originally EBV positive, driving B-cells to proliferate. This increased proliferation drives cellular mutations of tumour suppressors and oncogenes. Supporting this theory, late-onset PTLDs have been associated with mutations in RAS, p53, c-myc and BCL-6 [209, 210].

1.9 Treatment of Post-Transplant Lymphoproliferative Disease

PTLD is associated with a high mortality rate. Despite the range of treatments available, over 30% of patients with refractory disease die from PTLD [211, 212]. The main treatments for

PTLD include depletion of CD20 positive B-cells by the chimeric monoclonal antibody Rituximab, and reducing immunosuppression with anti-viral therapy.

Rituximab has been used against both HSCT- and SOT-derived PTLD, with varying responses. Phase II trials using Rituximab as a single agent to treat PTLD after SOT have shown response rates between 42%-70% [213, 214]. This range of response is likely due to time of diagnosis and type of organs transplanted.

However, these patients often relapse, and the mortality rate associated with this treatment is 50-80%, with the average survival time being 35 months [204, 215]. Furthermore, rituximab is associated with a diverse range of side effects, including: immediate hypersensitivity to the infusion, nausea, vomiting, fever and chills, systemic effects of tumour degradation, neutropenia, and profound B lymphocyte depletion with increased susceptibility for cytomegalovirus (CMV) reactivation. This clearly highlights the need for more effective and less toxic therapies.

Reduction in immunotherapy is commonly used as treatment for PTLD following SOT. In HSCT patients whose immune system has been fully ablated, reducing immunosuppression is often ineffective, as reconstitution of the donor derived immune system is not increased. Reduction in immunosuppression in 67 SOT patients resulted in 45% of patients responding, however half of these relapsed [216]. Treatment led to graft rejection in 32% of these patients, highlighting the limitations of this therapy. Nevertheless, reduction in immunosuppression has clear benefit to some PTLD patients – namely those that develop early-onset PTLD following SOT. Reducing immunosuppression in early-onset PTLD (less than one year after transplant) gives response rates of 80%, yet responses in late-onset PTLD are as low as 6% [217]. This difference is likely due somewhat to the different EBV gene expression in early- and late-

onset PTLD. As early-onset PTLD commonly presents with a latency III profile, reducing immunosuppression allows the immune system to target immunodominant viral antigens. Later stage PTLD, with limited EBV gene expression, has reduced immunogenicity. This suggests that treating PTLD in a stratified manner, according to the time of disease onset, and type of transplant, could have beneficial effects in the clinic.

There are other treatments for PTLD, including, radiotherapy, cytokine therapy and surgery. Chemotherapy combined with Rituximab has shown to increase the overall response to therapy; however it is associated with treatment related toxicity. In a retrospective study of 35 patients, overall response was increased by 6% when patients received Rituximab and chemotherapy rather than Rituximab alone. Nevertheless, 26% of patients receiving chemotherapy died of toxicity [218]. Chemotherapy is often used to treat PTLD patients after SOT but not after HSCT as these patients have already been treated with extensive chemotherapy and thus treatment related toxicity would likely be high [219].

EBV-associated tumours can also be targeted specifically due to the presence of the EBV genome, however identifying a clear way to deliver EBV specific drugs to their target T-cells has been challenging. Ghosh et al. have performed a phase I/II trial, in which they combined lytic inducers with anti-viral drugs. Anti-viral drugs such as acyclovir are phosphorylated by viral derived kinases. Phosphorylated acyclovir is a competitive nucleoside analogue that gets incorporated into viral DNA during replication and from here inhibits viral DNA polymerase activity [220]. Therefore anti-viral drugs are only toxic to lytically replicating virus. Consequently, increasing the frequency of lytic replication in infected cells should increase the therapeutic effect. They found this combination to be effective, with 10 of 15 patients

showing anti-tumour responses. However, toxicity due to rapid tumour cell lysis occurred in three patients [221].

1.9.1 Immunotherapy to treat PTLD

EBV-related tumours can be treated with immunotherapy because they express viral antigens and so can be targeted specifically. PTLD in particular is a target of immunotherapy, as it occurs in an immunocompromised host and expresses viral target antigens. As such, there are numerous epitopes that could be targeted.

1.9.2 DLI and CTL Infusion

DLI has been clinically utilised for relapse following HSCT in multiple haematological diseases and for the treatment of PTLD [222]. DLI can abolish PTLD, however not without considerable risk of developing GvHD due to mismatched HLA alleles [101, 223, 224]. A safer form of DLI has been developed; donor derived EBV-specific CTLs.

CTLs are expanded *in vitro* by reactivation of PBMCs using donor-derived LCLs or specific EBV peptides. Reactivated T-cells can therefore be polyclonal for a variety of EBV epitopes, increasing the immune response. Toxicity is reduced as selecting for EBV-specific CTLs reduces the risk of GvHD. Adoptively transferred EBV specific CTLs can survive *in vivo* (8 years), proliferate, and reduce EBV load in 20% of patients who have EBV reactivation following transplant [225, 226]. Furthermore, when used as a prophylactic treatment, EBV-specific T-cell infusions resulted in 0/58 post-transplant patients developing PTLD, compared to 11.5% of (historical) control patients [192, 227]. In a fifteen year summary of all EBV-

CTL infusions for the treatment of PTLD, 67% of patients who had received a HSCT and 51% of patients who had received a SOT responded [228].

One major drawback of EBV-CTL therapy is the production time. To stimulate and expand enough CTLs for clinical relevance takes between three to four months, as LCLs need to be generated and then used to expand EBV-specific T-cells to clinically relevant numbers. To overcome this, third party EBV-CTL bio-banks have been established, which eliminate this lag to treatment [229, 230]. The third party EBV-CTLs were used in a phase II trial to treat patients following SOT, as donor blood is often unavailable. They have shown that after six weeks 52% of patients respond, in a poor prognosis group unresponsive to conventional treatments. CTLs were selected on a best HLA match basis and patients receiving closer HLA matches had stronger responses. At a T-cell infusion rate of 2 million T-cells per kg, once a week for four weeks, no toxicity was noted [229]. Despite these advances, 48% of patients do not respond to this therapy. Efforts are thus on-going to increase the cure rate following PTLD. One such effort includes the use of genetic engineering of EBV-CTLs to render them resistant to immune suppressive drugs. Ricciardelli et al. have developed calcineurin resistant EBV-CTLs, which allow them to persist in the face of drug-induced immune suppression, such is the case for SOT patients [231]. CTLs engineered with this construct survived better than control CTLs when tested against human EBV positive B-cell lymphomas in the presence of steroids *in vivo*. Furthermore, tumour bearing mice that were treated with these engineered T-cells had significantly improved survival than mice treated with control EBV-CTLs [232].

1.9.3 TCR Gene Transfer for Treatment of PTLD

TCR gene transfer for the treatment of PTLD has not yet been fully explored. Limited MHC class I restricted EBV specific TCRs for the treatment of a range of EBV associated malignancies have been isolated. Whilst these may be useful in the treatment of PTLD they have not been tested in such setting to date. Of the studies performed, early studies investigating EBV specific TCR transduced T-cells showed limited efficacy. In 2001, Orentas et al. isolated a class I restricted LMP2 specific TCR for the treatment of EBV positive malignancies which express a latency II gene programme. Through *in vitro* functional studies the group demonstrated limited IFNγ production in response to peptide exposed target T-cells [233]. Furthermore, Jurgens et al. have isolated TCRs restricted through HLA A2, A23, and A24, specific for LMP2. T-cells transduced with these TCRs were able to lyse a proportion of LCLs *in vitro*, albeit only at high effector: target ratios and again, only when targets were pre-exposed to peptide [234]. Recent studies have showed more convincing anti-tumour responses, with Zheng et al. demonstrating the efficacy of LMP2 TCR gene transfer therapy. Here the TCR was restricted through the HLA allele A11 which is common in the Chinese population, in order to treat a large proportion of nasopharyngeal carcinoma patients. Transduction with this TCR led to proliferation, cytokine release and cytotoxicity to target T-cells both *in vitro* and *in vivo* [235].

Despite the proven importance of CD4+ T-cells in anti-tumour immunity, this T-cell subset has not been fully explored in the context of TCR gene transfer. Specifically, CD4+ T-cells have been suggested to enhance T-cell responses towards PTLD, and yet MHC class II restricted TCRs specific for EBV antigens have not been isolated [229, 236]. LMP2 specific MHC class I restricted TCRs have been transduced into CD4+ T-cells and shown to be

functional; transduced T-cells have shown tumour protection *in vivo* [235, 237]. However, to our knowledge, there has not been an MHC class II restricted gene transfer therapy that targets an EBV epitope.

1.10 Scope of thesis

This thesis focuses on the generation of an MHC class II restricted TCR gene transfer therapy for the treatment of PTLD and other EBV associated malignancies which express EBNA2. These include AIDs related lymphomas and DLBCLs. MHC class I restricted TCR gene transfer has been studied in depth but to date, reports on the feasibility and efficacy of MHC class II restricted TCR gene transfer are more limited.

As CD4+ T-cells respond to tumours in multiple ways, including producing cytokines to orchestrate both the adaptive and innate immune response, and directly recognising and killing tumour cells, I have isolated an *MHC class II* specific TCR, and transduced this into both CD4+ and CD8+ T-cells. By generating tumour specific MHC class II restricted T-cells we can improve the immune response to tumours through CD4+ T-cell activation. Furthermore, some tumours evade immune responses by down-regulating MHC class I, and therefore targeting *MHC class II* restricted epitopes increases our range of potential targets [238, 239]. We aim to isolate CD4+ T-cells which, through a range of *in vitro* studies, show therapeutic potential. We plan to clone TCRs from these cells intro retroviral constructs to allow for the transduction of healthy donor T-cells. Transduced T-cells will be subjected to a range of *in vitro* and *in vivo* assays to establish their therapeutic potential. Finally, we aim to explore EBV protein expression in PTLD to determine if there are any other EBV proteins which would make good targets for TCR gene transfer therapy for the treatment of this disease.

CHAPTER 2

2 Materials and Methods

2.1 Tissue culture

2.1.1 Culture mediums

Standard Culture Media: RPMI 1640 media (Sigma) supplemented with 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 10% fetal bovine serum (FBS) (Biosera)

T-cell media: standard culture media supplemented with 1% pooled human serum (TCS Biosciences) and 100U/ml Proleukin (IL2) (Novartis).

CD4 cloning media: standard culture media supplemented with 1% HuS, 50U/ml IL2 and 30% filtered supernatant harvested from the Monkey Leukocyte Antigen 144 (MLA 144) cell line.

Phoenix media: DMEM (Sigma) supplemented with 10% FCS, 2mM L-glutamine, 100IU/ml penicillin and 100µg/ml streptomycin.

Burkitt's media: RPMI 1640 media supplemented with 10% B-cell serum (Life Technologies), 1% pyruvate (Sigma), 0.1% alpha-TG (Sigma), 2mM L-glutamine, 100IU/ml penicillin and 100µg/ml streptomycin.

Freezing media: RPMI 1640 containing 2mM L-glutamine, 20% FBS and 10% DMSO (Fischer Scientific).

SOC: SOB (Becton Dickinson) media, supplemented with 2% filter sterilised 20% glucose.

MLA 144: Supernatant from the Monkey Leukocyte Antigen 144 cell line (lymphosarcoma) cultured for 2 weeks in standard media was filtered through 0.45µM filters and stocks frozen at -20 °C.

MACs buffer: PBS with 0.5% Bovine Serum Albumin (BSA) and 2.5mM Ethylenediaminetetraacetic acid (EDTA).

TAE: 1x TAE made up from 50x TAE Ultra Pure buffer (Geneflow) in deionised H2O.

Coating buffer: 10x stock was made of 1.36g Sodium Carbonate (Sigma), 7.35g Potassium bicarbonate (Sigma) and 100ml H_2O. The buffer was adjusted to pH9.2 with 1M HCL (Sigma) or 1M NaOH (Sigma).

Blocking buffer: 5g of BSA (Sigma) and 250µl of tween (Fischer Scientific) was added to 500ml of PBS (Thermo Scientific).

2.1.2 Generation and maintenance of LCLs

Lymphoblastoid cell lines (LCLs) were generated by *in vitro* transformation of B cells with EBV. To generate LCL, 4ml supernatant from virus producer lines (B95.8 or Ag876 EBV strains) was spun at 509 x g for 5 min to pellet cell debris. Supernatant was then filtered through a 0.45µm syringe filter and combined with 0.5ml FBS (Biosera) and used to re-suspend 5-10 million pelleted PBMCs. After an overnight incubation at 37 °C 5% CO2, PBMC/virus mix was spun at 800 x g for 5 min. Supernatant was discarded and PBMCs resuspended in 2ml culture media supplemented with 1µg/ml cyclosporin A (Sandoz) in a 24 well plate. Cells were split when they had proliferated sufficiently and maintained in culture media.

The LCLs used were either generated for this study or had been previously made during earlier work and stored in liquid nitrogen. Frozen cell lines were thawed as described below.

2.1.3 Cryopreservation

For cryopreservation, cells were centrifuged at 800 g x 5 min to obtain a viable pellet. Cells were resuspended in freezing media and transferred to a sterile 1.8ml cryovial (Thermo Scientific, Nunc). They were immediately transferred to a -80 °C freezer in a 'Mr Frosty' – a container containing isopropanol that is designed to lower the temperature of vials contained therein at a rate of -1 °C/min. When at -80 °C, cells were transferred to liquid nitrogen for long term storage.

2.1.4 Cell restoration

Cells were removed from nitrogen into a 37 °C water bath to thaw. Immediately after thawing, cells were suspended into their relevant media and washed twice via centrifugation to remove freezing media. Cells were then transferred to an appropriate culture flask or plate and incubated at 37 °C and 5% CO2.

For T-cell clones, one million cells were recovered from nitrogen as above and resuspended into a well of a 24-well plate. The well contained 1 million allogeneic PBMCs from three donors that had been Phytohemagglutinin (PHA) (Life Technologies)-treated (10µg/ml) overnight and $1x10^5$ HLA matched LCLs pre-exposed to cognate peptide (Alta Biosciences). Both PBMCs and LCL had been previously irradiated at 4000 rads.

2.1.5 Mycoplasma testing

Cell lines were routinely tested for the presence of mycoplasma. 200µl of cell supernatant was centrifuged at 800 x g for 5 min. Of this, 15µl was added to a 96 well optiplate, with 15µl room temperature mycoalert reagent (Cambrex) and mixed by pipetting. This was counted on TopCount NXT^{TM} microplate scintillation and luminescence counter (Perkin Elmer) for 1 second per well. Subsequently, 15µl of mycoalert substrate was added and the count was repeated. If the second count was greater than the first the culture being tested was mycoplasma positive and good laboratory practise was used to control the infection.

2.1.6 Maintenance of adherent cell lines

Phoenix cells were maintained in appropriate media. When cells reached between 75% and 100% confluencey, they were split. To split adherent cells, media was removed and cells were washed with PBS. 0.05% trypsin (Gibco) diluted in PBS was added to the cells. They were then incubated until they entered suspension. The trypsinisation reaction was then inhibited from proceeding further by the addition of appropriate media containing serum. The cells were subsequently counted and seeded at lower density.

2.2 Cloning EBV-specific T-cells

2.2.1 Donors & Consent

All blood donors used throughout this thesis were healthy volunteers who had provided written informed consent to participate in the study. The work was carried out under ethical approval by the South Birmingham Local Research Ethics Committee (07/Q2702/24).

2.2.2 Isolation of CD4+ T-cells from healthy donors

Peripheral blood mononuclear cells (PBMCs) from blood of consenting, healthy EBV carriers with known HLA types were isolated by density centrifugation using Lymphoprep (Axis-Shield). Blood was mixed with RPMI at a 1:1 ratio and layered onto lymphoprep. This was centrifuged at 800 x g for 30 min (no brake), and the mononuclear cells were isolated from the interface of plasma and lymphoprep. The harvested PBMCs were centrifuged in RPMI at 800 x g for 10 min (low brake), then washed again with RPMI but centrifuged at 600 x g for 10 min (low brake). A third wash in RPMI was conducted at 400 x g for 5 min (high brake) and resuspsended in culture media. CD8+ cells were subsequently depleted using CD8+ dynabeads (Invitrogen) according to the manufacturer's instructions.

2.2.3 Reactivation of PRS specific CD4 T-cells and T-cell cloning

CD8-depleted PBMCs were stimulated by addition of 5μM epitope peptide (either the EBNA2 derived peptide, "PRS" (PRSTVFYNIPPMPLPPSQL) or the BZLF1 derived peptide, "LTA" (LTAYHVSTAPTGSW)) for one hour. The cells were subsequently washed in standard media to remove the peptide and cultured in a 24-well plate at $1x10^6$ cells/ml. One week later, the PBMCs were harvested and restimulated for three hours with 5μM of the same epitope peptide. Responding cells were isolated using an IFNγ secretion assay (Miltenyi Biotec) according to the manufacturer's instructions. Captured cells were plated out at 0.3 and 3 cells per well of 96-well plates in a limiting dilution cloning protocol as previously described [240]. From 2 weeks, Growing microcultures were screened for response to peptide by IFNγ ELISA from plates where less than a third of wells had growing cells.

2.2.4 Expansion of T-cell cultures

PRS-specific T-cell cultures were expanded from 200 µl volumes into 2ml CD4 cloning media containing 10^5 peptide-pulsed γ-irradiated (4000 rads) LCLs and 10^6 γ-irradiated (4000 rads) phytohemagglutinin-treated (10µg/ml) mixed allogeneic buffy coat feeder cells (Birmingham National Blood Service).

2.2.5 Maintenance of T-cells

T-cell clones were maintained in CD4 cloning media. Clones were fed twice a week, or when the media appeared exhausted. When cells reached 1 million per ml, cells were split into two wells. Clones were maintained by the addition of 1 million irradiated (4000 rads) healthy donor PBMCs from three donors every two weeks or when necessary.

Transduced T-cells were maintained in T-cell media. Media was changed every three to four days. Cells were split when they reached between one and two million cells per ml.

2.3 Functional analysis T-cell clones

2.3.1 IFNγ ELISA

Standard ELISAs involved pre-exposing LCLs to 5µg peptide or DMSO (unless otherwise stated) for 90 min and washing three times with standard media. LCLs and T-cells were co-cultured overnight at ratios stated at 37 °C 5% CO2. The following day, the level of IFNγ released into the supernatant by responding T-cells was determined using the Thermo Scientific IFNγ ELISA protocol. Here, primary IFNγ antibody was diluted 1 in 1351 with ELISA coating buffer and 50 µl was added to each well of a 96 well Maxisorp plate (Nunc). The plate was incubated at 4°C overnight and blocked with 200µl blocking buffer the

following day. This was washed off with PBS and 100µl of test supernatant or IFNγ standard was added. IFNγ standard was made by double dilutions of IFNγ from 20,000 pg/ml to 0pg/ml in RPMI. After two hour incubation at room temperature, the wash was repeated and the secondary, biotin labelled IFNγ antibody was added to each well (50µl of a 1 in 1351 dilution in blocking buffer). This was incubated at room temperature for one hour and then the plate was washed in PBS. 50µl of Extravidin (Sigma) was then added to each well and the plate incubated at room temperature for half an hour. Following this, the plate was washed and 50µl of TMB (Life Technologies) was added to each well. When the standard had developed, 50µl of 1M HCL was added to each well and the plates were read on the Biorad iMark microplate reader.

For peptide titrations, target LCLs were exposed to ten-fold dilutions of peptide, ranging from 5µM to 5pM and incubated at 37ºC/5%CO_2 for 90 min re-suspending cells every 20-30mins. Peptide exposed LCLs were washed five times at 400 x g for 5 min in LCL media prior to use.

Where CD4 and CD8 T-cells were analysed separately, the relevant subsets were isolated through negative selection using CD4 or CD8 dynabeads (Dynal), as per the manufacturer's instructions. After depletions, a sample of each cell population was stained with both anti-CD4 and CD8 antibodies to assess purity.

2.3.2 MHC class II Tetramer staining

$1x10^6$ T-cells were washed in 500µl HuS. 0.5µl relevant HLA class II tetramer /peptide (made by Eddie James, Benaroya Research Institute, Seattle) was added in 100µl HuS and cells incubated for 2 hours at 37°C in the dark with frequent re-suspension [187]. After three washes in cold MACs buffer, cells were stained with anti-CD4 and anti-CD8 antibodies

conjugated to various fluorophores (see Table x) for 20 min at 4°C. After washing, cells were fixed in 500µl 2% paraformaldehyde (PFA – Fischer Scientific) before analysis on an Epics flow cytometer (Beckman Coulter). All data were processed using Flowjo software (TreeStar) version 7.6.5.

2.4 Construction of retroviral plasmid

2.4.1 RNA extraction from T-cell clones

RNA was isolated from PRS and LTA-specific T-cell clones following the RNAeasy minikit protocol (Qiagen), as per the manufacturer's instructions. RNA was either used immediately or stored at -80 °C.

2.4.2 Rapid Amplification of cDNA ends

TCR alpha and beta genes were amplified using gene specific primers, following the SMARTer RACE cDNA Amplification Kit and Advantage 2 PCR kits (Clontech) according to recommended protocols.

Alpha primer: 5'-TAG GCA GAC AGA CTT GTC ACT GGA TT-3'

Beta primer: 5'-CGA CCT CCT TCC CAT TCA CCC AC-3'

Cycling parameters for the PCR reaction were as follows: 5 cycles of 94 degrees 5 sec, 72 degrees 3 min. 5 cycles of 94 degrees 5 sec, 68 degrees 10 sec, 72 degrees 3 min. 27 cycles of 94 degrees 5 sec, 65 degrees 10 sec, 72 degrees 3 min, 4 degree hold.

Resulting DNA was ran on a 1% ultra pure agarose (Invitrogen) gel. Bands of the correct size (600-800 bp) were cut out and DNA was isolated using the Qiagen Gel Extraction kit and corresponding protocol.

2.4.3 DNA cloning

The Alpha and beta TCR gene sequences were cloned into TOPO TA vectors (Invitrogen) in accordance with the manufacturer's instructions. 4.5µl of DNA was added to 0.5µl of the TOPO vector. This was incubated on at room temperature for 20 min and then put on ice. 1µl of this mixture was added to 50µl of competent cells on ice and left for 20 min. Following this the cells were heat shocked at 42 °C for 30 seconds and then put back on ice for 2 min. After the addition of 250µl SOC media the mixtures were shaken in a 37 °C incubator for one hour. This was then spread onto pre-warmed agar + 100µg/ml ampicillin (Roche) plates which were incubated at 37 °C overnight. The following day, individual colonies were picked and added to 50µl H_2O and re-plated onto a new agar plate with 100µg/ml ampicillin. The bacteria in H_2O was heated to 100 °C for 10 min. 5µl of this was added to a PCR mastermix to determine which colonies were successfully transformed with the TOPO vector by amplifying DNA from M13 primers. The PCR mixture contained 5µl DNA, 1x PCR buffer with Mg^{2+}, 200µM dNTPs, 0.5mM M13 forward and 0.5mM M13 reverse primers, 1 unit of granzyme.

Fast Start Taq and was made up to 10µl with H_2O. The PCR cycles used were as follows: 94 °C 5 min, 40 cycles of 94 °C 20 sec, 50 °C 30 sec, 72 °C 1 min, then 72 °C for 7 min and a 4°C hold. The agar plate with the re-plated bacteria was incubated at 37 °C 5% CO_2 for 16 hours and then kept at 4 °C.

PCR reactions were run on a 1% agarose gel (Invitrogen) and colonies which gave bands at the expected size of 800 bp were re-picked. These were amplified in 5ml LB broth + 100µg/ml ampicillin for 16 hours in a 37 °C shaking incubator. DNA from these cultures was subsequently isolated using the Qiaprep Mini Prep kit and corresponding protocols.

2.4.4 DNA sequencing

10µl reactions with 1-10 ng of DNA and 3.2 pg of M13 forward or reverse primer were sent to the functional genomics laboratory at Birmingham University for sequencing. Here they were supplemented with 0.5µl Big Dye and amplified by PCR reaction (96 °C for 1 min followed by 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, 60 °C 4 min) ran on the capillary sequencer ABI 3730.

2.4.5 Vector assembly

The TCR sequence was constructed by PCR with DNA overlap extension using NEB Phusion High Fidelity Polymerase. Both alpha and beta variable fragments were linked to the previously codon optimised alpha and beta constant fragments. These full length alpha and beta sequences were assembled into a single gene construct separated by a 2A linker from porcine Teschovirus.

Cycle parameters: 98°C 30 sec, 35 cycles of 98°C 5 sec, *°C 20 sec, 72°C 15 sec. Then 72°C 5min, 4° hold.

*primers variable beta, constant beta at 66°C, variable alpha and constant alpha at 68°C.

This was then inserted into an MP71 retroviral vector (provided by C. Baum, Hannover, Germany) using BamH1 and Not1 restriction sites and the Quick Ligation (NEB) kit according to the manufacturer's instructions (see Figure 3.10 for cloning strategy). Vectors were subsequently amplified through bacterial transformation, as described in DNA cloning.

2.4.6 Transformation

TOP 10 (Invitrogen) competent cells were transformed with the MP71-TCR vector by heat shock. Briefly, competent cells were thawed on ice and DNA (100pg-10ng) was added for half an hour. The competent cells were then given a 30 second heat shock at 42 °C and returned to ice. After the addition of 250µl SOC media, cells were incubated in a shaking incubator at 37 °C for one hour, before being plated out on agar plates containing 100µg/ml ampicillin and incubated overnight. The following day, individual colonies were picked to grow up in LB broth with ampicillin for 16 hours.

Vector DNA was obtained through a mini prep (Qiagen) and the MP71 vector constructs were analysed by restriction enzyme digestion (BamH1 and Not1, NEB) and DNA sequencing.

2.4.7 Restriction enzyme digests

For vector construction, 1µg of DNA was digested with 10 units (1µl) of relevant restriction enzyme, in the appropriate buffer, in a reaction volume of 25-50µl for one hour at 37 °C.

Following digestion of the vector for insertion of the TCR alpha and beta chain sequences, the vector DNA was treated with 10µl Calf Intestinal Phosphatase (NEB) for one hour at 37 °C immediately after the digest, to prevent vector re-ligation.

Digested DNA was subsequently run on an agarose gel (0.5-2% ultra pure agarose (Invitrogen) depending on DNA size), in 1xTAE buffer with 1/1000 dilution of sybr green (Invitrogen) at 100V.

If the DNA was to be extracted from gel, the DNA band would be cut out and DNA isolated with a gel extraction kit (Clontech), as per manufacturer's instructions.

2.4.8 Vector amplification

TCR-MP71 vectors were transformed into One Shot® TOP10 chemically competent E-coli cells (life technologies), as described in section 2.4.3. Colonies were picked the from agar + 100µg/ml ampicillin plates the following day and cultured in 5ml LB broth + 100µg/ml ampicillin for 16 hours. DNA was extracted for sequencing from 1ml of this culture by Qiagen Mini Prep as per manufacturer's instructions. Cultures which contained the TCR-MP71 DNA were amplified by being added to 300ml of LB broth + 100µg/ml ampicillin and incubated at 37 °C overnight. DNA was isolated using the endo-free maxi prep kit (Qiagen) as per manufacturer's instructions.

2.5 Transduction

Recombinant retrovirus was generated using the Phoenix A packaging cell line (Nolan Laboratory, Stanford University, Stanford, CA). Phoenix cells cultured in phoenix media and seeded on day 0 to reach 50 – 80% confluence on day 1. Phoenix cells were then transfected in penicillin- and streptomycin- free media with the appropriate vector using fugene (Promega), as per manufacturer's instructions. PBMCs were stimulated on day 1 with 300U/ml IL2, 30ng/ml anti-CD3 (OKT3, eBioscience) and 30ng/ml anti-CD28. On day 2, media on the phoenix cells was replaced with fresh media. On day 3, PBMCs were transduced with recombinant retrovirus harvested in the supernatant from the phoenix cells as previously described [241]. Briefly, non-tissue culture treated plates were coated for three hours in retronectin (30µg/ml) (Takara) then blocked using 3% w/v BSA in PBS. After three washes with PBS, viral supernatant was applied and plates spun for 2 hours at 2000 x g at 37°C. The

plate was washed once with PBS, PBMCs added in TCM and spun down at 800 x g for 5 min. The TCM was changed the following day and T-cells analysed for transduction two days later.

2.6 Functional analysis of transduced cells

2.6.1 Flow cytometry

$1x10^5$ cells were resuspended in 40µl PBS and stained with 1.5µl of a 1in100 dilution of LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Life technologies) in the dark at room temperature for 20 min. After one wash in MACs buffer, cells were surface stained with appropriate antibodies (see Table 2.3) in 100µl MACs buffer and placed on ice in dark for 30 min. Cells were washed three times in cold MACs buffer and analysed on the Epics flow cytometer (Beckman Coulter) or BD LSR II flow cytometer. All data were processed using Flowjo software (TreeStar) version 7.6.5.

2.6.2 Intracellular Cytokine Staining (ICS)

$1x10^6$ T-cells were either co-cultured with $5x10^5$ peptide pulsed LCLs or unmanipulated LCLs for an hour in TCM, or left unstimulated. Cultures were then washed twice at 400 x g for 5 min before being resuspended in 1ml TCM containing 10µg/ml Brefeldin A (BFA, Sigma) and plated out into a 48 well plate. Plates were incubated overnight at 37°C. The following day, cultures were washed in MACs buffer at 800 x g for 5 min, the supernatant discarded and anti-CD4 and anti-CD8 antibodies (see Table 2.10) were added to the residual buffer of relevant tubes. Cultures were incubated for 20 min at 4° in the dark. After washing in MACs buffer, all cells were resuspended in 100µl 4% PFA and incubated at 25°C for 15 minutes in the dark. Following a wash in PBS, 100µl 0.5% saponin (sigma) was added to each tube and tubes were then incubated at 25°C for 5 minutes in the dark. Intracellular

antibodies were then added and after 30 minutes incubation at 25°C in the dark, cells were washed, resuspended in MACs buffer and analysed by flow cytometry as above.

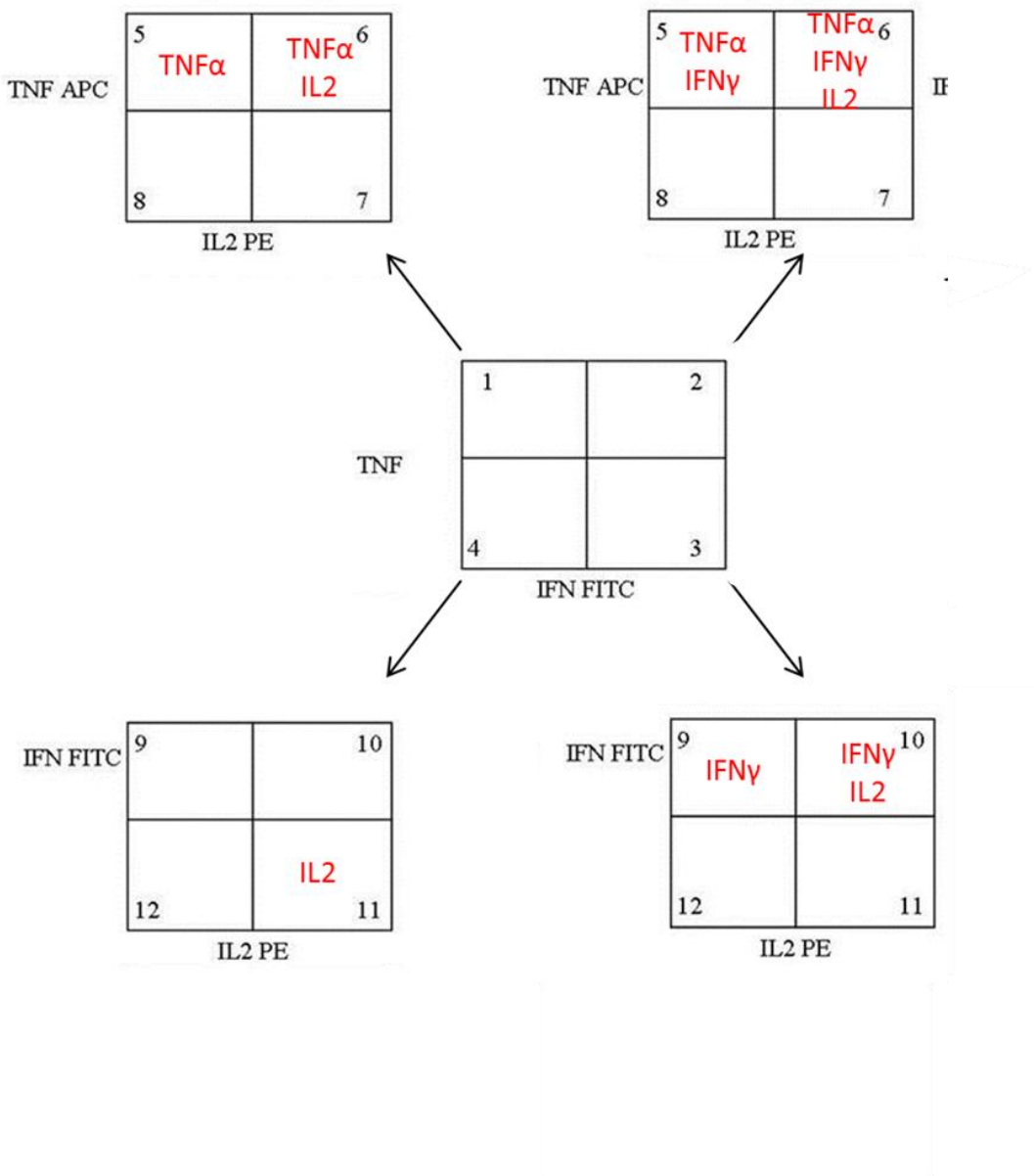


Figure 2.1 Gating strategy used to determine which T-cells produce one, two and three cytokines. Cells were gated on single cells by gating out doublets using FSC-H amd FSC-A. Lymphocyes were gated using FSC/SSC. Live cells were gated using LIVE/DEAD® Fixable Violet Dead Cell Stain and CD4+ or CD8+.

Gate 1 was drawn and subsequent gating is drawn from gate 1 quadrants. Gates which contain cells which produce one, two or three cytokines are indicated by the red labels.

2.6.3 CD107a analysis

T-cells were co-cultured with HLA DR52b-matched LCLs pre-exposed to 5µg/ml peptide or DMSO at a 4:1 target: effector ratios. Also in the culture was 0.25µg of FITC-conjugated anti-CD107a (BD Biosciences) and 1x Monensin (Sigma). After 6, 12 and 24 hour incubations cells were washed in MACS buffer and stained with a viability dye as described above, followed by anti-CD4 and CD8 antibodies. Samples were analysed on the BD LSR II Flow Cytometer. All data were processed using Flowjo software (TreeStar) version 7.6.5.

2.6.4 Proliferation

Autologous LCLs were irradiated at 4000 rads then exposed to 5µg/ml PRS or DMSO for 90 min and washed twice in culture media. T-cells were labelled with 1µM CFSE (Invitrogen) and co-cultured with DMSO and peptide exposed LCL for 5 days at a responder: stimulator ratio of 1:10. On day 5, surface staining for CD4 and/or CD8 was performed (see above) and samples analysed as described above.

2.6.5 DC maturation assay

Autologous immature DCs (iDCs) were isolated from PBMCs by leaving PBMCs undisturbed in a flask for 2 hours, after which non adherent cells were removed. The adherent cells were differentiated into immature-DCs by culturing in standard culture media supplemented with 50ng/ml IL4, 50ng/ml GMCSF (Peprotech) for 5 days. On day 5, iDCs were exposed to epitope peptide or not for 90 min and co-cultured for two days with T-cells at a 1:1 T-cell: DC ratio, in TCM supplemented with 10U/ml IL2. iDCs in TCM + 10U/ml IL2 acted as the negative control, and iDCs matured by culture in TCM with 10U/ml IL2 and 10ng/ml IL1β, 100ng/ml IL6, 20ng/ml TNFα, 50ng/ml IL4, 50ng/ml GMCSF (peprotech) were

the positive control. On day 7, cultures were analysed by flow cytometry for live cells using the LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Life technologies) , and for the surface expression of CD3, CD83, HLA DR, CD86 and CD40 (for antibodies, see Table 2.3).

2.6.6 Chromium Release Assay

LCL Targets were labelled with 50µCi ^{51}Cr (Perkin Elmer) for two hours whilst being exposed to 5µg/ml peptide or not. After washing with culture media, targets were co-cultured with T-cells in triplicate wells of a 96-well V-bottom plate at defined effector: target ratios as described in the results. The percentage of ^{51}Cr released into the culture supernatant after 5 and/or 12 hours incubation was measured using the Cobra II Gamma Counter (Packard). Percent specific lysis was calculated as follows: (target release-spontaneous release)/(maximum release - spontaneous release)*100.

2.6.7 Outgrowth assay

For outgrowth assays, autologous LCLs were exposed to 5µg/ml peptide or not for 90 min. LCLs were then washed five times in culture media and plated out in a sterile 96 well U bottom plate at doubling dilutions from $1x10^5$ to 97 LCLs per well. $1x10^5$ T-cells were subsequently washed and added to wells containing the LCL. Control wells were also set up containing LCL or T-cells alone and all cells were then incubated at 37 °C 5% CO2 for four weeks. Media was changed weekly. The outgrowth of LCL cells was assessed at 4 weeks visually.

2.7 *In vivo*

All work was carried out under a Home Office approved Project and Personal Licence.

2.7.1 Generation of Luciferase transduced LCLs

The pMSCV luciferase plasmid contained a puromycin resistance gene. This was amplified in Phoenix A cells and harvested as described above. 2ml Retronectin (30µg/ml) was added to the wells of 6 well non tissue culture treated plates and these were left at 4 °C overnight. The following day, the wells were blocked with 2.5ml of 2% BSA for 30 min. After three washes in PBS, 2ml supernatant harvested from the Phoenix A cells was added to each well and the plates were centrifuged at 2000 x g for 2 hr at 37 °C. LCLs were seeded at a concentration of $3x10^6$/ml in standard culture media, and added to retrovirus coated plates. These were centrifuged at 800 x g for 4 min and then incubated at 37 °C 5% CO_2.

After four days incubation, standard culture media was supplemented with 1µg/ml puromcyin (Sigma), to select transduced LCLs.

2.7.2 Injection of LCLs

LCLs were washed in PBS and resuspended in PBS at the required concentration. Cells were kept on ice and 200µl was injected, subcutaneously into the flank or intraperitoneally, into mice. For subcutaneous injection, mice were anaesthetised and shaved prior to injection.

2.7.3 Injection of T-cells

T-cells were washed and resuspended in RPMI at the required concentration. Cells were kept on ice and a maximum of 200µl was injected intravenously into the mouse tail vein.

2.7.4 Injection of Decitabine

0.25mg/kg decitabine was injected into mice once on day 0 and twice on days 1, 2 and 3, intraperitoneally.

2.7.5 Peritoneal Wash

LCLs were retrieved from culled mice by intraperitoneal washing. 5ml PBS was injected into the mouse peritoneum. The peritoneum was subsequently massaged to dislodge LCLs and the PBS was isolated by syringe.

2.7.6 *In vivo* monitoring

Mice were monitored three times a week for tumour size by caliper (for subcutaneous tumours) and once a week by bioluminescence imaging (IVIS Spectrum, Caliper Life Sciences) (for IP tumours). For IVIS imaging, mice were injected with 10µl luciferin per gram of body weight. After five minutes, mice were anaesthetised with Isoflurane and imaged.

Mice were monitored three times a week for signs of distress including weight loss, lethargy, ruffled fur, disinterest in surroundings, body condition, changes in colour of feet and ears, bulging cheeks. Mice were culled by a schedule 1 method when showing signs of distress or at experimental end points.

2.8 Immunohistochemistry

2.8.1 TPA induction of Akata cells

EBV positive Akata cells were thawed and cultured in Burkitt's media, at $1x10^6$/ml. To induce Akata cells into lytic cycle, cells were seeded at $0.5x10x10^6$/ml in a 24 well plate to which 20µg/ml Tetradecanoyl Phorbol Acetate (TPA) (New England Biolabs) and 3µl/ml of 1M Sodium Butyrate (Sigma) were added. After incubation at 37 °C overnight at 5% CO2, cells were washed and plated out at $2x10^6$ per well of a 6 well plate, in 6ml Burkitt's media. After 48 hours incubation, cells were spun down onto microslides using the cytospin preparation method.

2.8.2 Cytospin preparation

$2x10^6$ cells were spun onto each X-tra adhesive microslide (Suripath Europe) in cytospin preparation. Cells were resuspended in PBS and fixed with 4% formaldehyde (Sigma Aldrich). Slides were assembled into cytoclips (Thermo Fisher Scientific) and covered with filter cards (Thermo Fisher Scientific). Plastic Cytofunnel disposable sample chambers (Thermo Fisher Scientific) were placed on top of the filter before the metal clamp was closed. These coverslips were then placed into the cytospin 3 cytocentrifuge (Shandon) before cells were added via the funnel. Cells were spun onto the slides at 400 x g for 5 min and then air dried for 30 min. The slides of were either stained immediately or stored at -20°C until use.

2.8.3 Immunohistochemistry staining

Immunohistochemistry was performed on paraffin embedded tissue sections and cytospun cells. With ethical approval, PTLD biopsies were obtained from Human Biomaterials Resource Centre (HBRC) at the University of Birmingham and Nottingham Tissue Biobanks.

Paraffin embedded samples were de-waxed in histoclear (National Diagnostics) for 10 min and dehydrated in industrial methylated spirits for 5 min (Sigma Aldrich). Hereafter, the same methods were applied to cytospins and fixed tissue samples. Endogenous peroxide activity was blocked with 0.3% H_20_2 (Sigma Aldrich) for 15 min, followed by antigen retrieval as described: Citrate buffer (1.26g sodium citrate and 0.25g citric acid in 1L distilled water pH 6.0) was boiled in an 800 watt microwave for 10 min at high power and then slides were added. These were microwaved for 10 min at medium power, 10 min at low power, and then left at room temperature or 4°C until cool. After washing for 5 min in water, circles were drawn around the samples using an ImmEdge Hydrophobic Barrier Pen (Vector Laboratories Ltd) to prevent dispersion of antibodies. Slides were conditioned with PBS-Tween 20 (0.1%) (PBST) for 5 min before blocking with 5x Casein blocking solution (Vector Laboratories Ltd). Primary antibodies were added and left at 4 °C overnight (see Table for list of antibodies and dilutions). The following day, slides were washed with PBST (3 x 3 in) before the addition of the secondary antibodies, and incubated at room temperature for 30 min. After three more washes with PBST, DAB substrate was added (ImmPACT DAB Substrate System, Vector Laboratories) for between 1-5 min for visualisation. Slides were washed in water for 5 min then stained by Mayer's haematoxylin (Sigma Aldrich) for 1 min. Slides were washed in warm water for 5 min before being placed in IMS for 15 min and histoclear for 10 min. DPX mounting solution (Sigma Aldrich) was utilised to attach cover slips to the slides.

2.8.4 In Situ Hybridisation

In Situ Hibridisation (ISH) was performed using RNA free equipment. Slides containing formalin fixed tissues were de-waxed in histoclear for 10 min and hydrated by two washes in 99% ethanol (Sigma Alrich) for 3 min. Slides were then washed in 95% ethanol for 3 min and

washed twice in water for 3 min each. The ImmEdge hydrophobic barrier pen was used to draw circles around tissues, then the slides were covered in proteinase K (Sigma Alrich) (5µg/ml) in 50mM TRIS/HCL buffer (pH 7.6) for 30 min at 37°. Slides were then washed twice in water for 3 min and dehydrated by washing in 95% ethanol for 3 min, followed by 99% ethanol for 3 min. Tissues were air dried before addition of the probe hybridisation solution (Vector). Coverslips were used to retain this solution whilst slides were incubated at 55 °C for 90 min. Slides were subsequently washed three times in TBS 0.1% Triton-x-100 (Sigma) for 3 min each. Tissue sections were blocked with rabbit serum (Dako) for 10 min before the addition of rabbit F(ab) anti FITC/AP (Vector) in TBS 3% BSA, 0.1% Triton-x-100. Slides were incubated for 30 min at room temperature. Slides were washed twice in TBS for 3 min before the addition of enzyme substrate and inhibitor (levamisole) (Vector) in 100 mM Tris/HCl, 50 mM MgCl2, 100 mM NaCl (pH 9.0). Slides were incubated overnight at room temperature, in the dark. The following day, slides were washed in TBST followed by water for 5 min. Tissues were counterstained in fast red (Vector)for 2-3 min and then dehydrated in IMS for 15 min before being cleaned in histoclear for 10 min and mounted, using DPX.

2.9 Primer Tables

Beckman Coulter (antibodies)	IMGT nomenclature	Sequence 5’ to 3’
TCR Vβ1	TRBV9*01 TRBV9*02 TRBV9*03	CAACAGTTCCCTGACTTGCAC
TCR Vβ2	TRBV20-1*03 TRBV20-1*04 TRBV20-1*05 TRBV20-1*06 TRBV20-1*07	TCAACCATGCAAGCCTGACCT
TCR Vβ3	TRBV28*01	TCTAGAGAGAAGAAGGAGCGC
TCR Vβ4	TRBV29-1*01 TRBV29-1*02 TRBV29-1*03	ACATATGAGAGTGGATTTGTCATT
TCR Vβ5.1	TRBV5-1*01 TRBV5-1*02	CTTCAGTGAGACACAGAGAAAC
TCR Vβ5.2	TRBV5-5*01 TRBV5-5*02 TRBV5-5*03 TRBV5-6*01 TRBV5-7*01	CCTAACTATAGCTCTGAGCTG
TCR Vβ6	TRBV7-3*04 TRBV7-3*05 TRBV7-8*01 TRBV7-8*02 TRBV7-8*03	GGCCTGAGGGATCCGTCTC
TCR Vβ7	TRBV4-2*02 TRBV4-3*01 TRBV4-3*02 TRBV4-3*03 TRBV4-3*04	TGAATGCCCCAACAGCTCTC
TCR Vβ8	TRBV12-3*01 TRBV12-4*01 TRBV12-4*02	ATTTACTTTAACAACAACGTTCCG
TCR Vβ9	TRBV3-1*01	AATCTCCAGACAAAGCTCAC
TCR Vβ10	TRBV21-1*01	TCCAAAAACTCATCCTGTACCTT
TCR Vβ11	TRBV25-1*01	ACCAGTCTCCAGAATAAGGACG
TCR Vβ12	TRBV10-3*01 TRBV10-3*02 TRBV10-3*03 TRBV10-3*04	TGACAAAGGAGAAGTCTCAGAT
TCR Vβ13.1	TRBV6-5*01	GACCAAGGAGAAGTCCCCAAT
TCR Vβ13.2	TRBV6-2*01 TRBV6-3*01	TGGGTGAGGGTACAACTGCC
TCR Vβ14	TRBV27*01	CTCTCGAAAAGAGAAGAGGAAT

TCR Vβ15	TRBV24-1*01	TCTCTCGACAGGCACAGGCT
TCR Vβ16	TRBV14*01	AGAGTCTAAACAGGATGAGTCC
TCR Vβ17	TRBV19*01	TCACAGATAGTAAATGACTTTCAG
TCR Vβ18	TRBV18*01	GAGTCAGGAATGCCAAAGGAA
TCR Vβ19	TRBV23-1*01	CCCCAAGAACGCACCCTGC
TCR Vβ20	TRBV30*01 TRBV30*02 TRBV30*04 TRBV30*05	TGAGGTGCCCCAGAATCTC
TCR Vβ21	TRBV11-2*03	TCCAACCTGCAAGGCTTGACGACT
TCR Vβ22	TRBV2*01 TRBV2*02 TRBV2*03	GAGAAGTCTGAAATATTCGATGATC
TCR Vβ23	TRBV13*01	GCAGGGTCCAGGTCAGGACCCCCA
TCR Vβ24	TRBV15*01 TRBV15*02 TRBV15*03	CCCAGTTTGGAAAGCCAGTGACCC
TCR Vβ constant		CGACCTCCTTCCCATTCACCCAC

Table 2.1 Primer sequences used for PCR of the variable beta TCR chain. All sequences are written in the 5' – 3' orientation.

Primer	Sequence
C15 Vα F	GCGCGGCCGCACCATGAGGCAAGTGGCGAGAGTG
C15 Vα R	GGGTCGGGGTTCTGGATGTTTGCTAAAACCTTCAGCCTGG
C15 Vβ F	CGTGGAGGAAAACCCTGGCCCCATGAGAATCAGGCTCCT GTGCTGTG
C93 Vα F	GCGCGGCCGCACCATGAAGACATTTGCTGGATTTTCGTTC C
C93 Vα R	GGGTCGGGGTTCTGGATGTTTGGACTGACCAGAAGTCGG G
C93 Vβ F	GTGGAGGAAAACCCTGGCCCCATGAGCAACCAGGTGCTC TGC
Vβ R	GAACACGTTCTTCAGGTCCTCTACAACTGTGAGTCTGGTG CC
C105 Vα F	GCGCGGCCGCACCATGGTGAAGATCCGGCAATTTTTG
C105 Vα R	GGGTCGGGGTTCTGGATGTTTGGGTTGATAGTCAGCCTGG
C105 Vβ F	GTGGAGGAAAACCCTGGCCCCATGATGCTCTGCTCTCTCC TTG
Cb F	GAGGACCTGAAGAACGTGTTC
Cb R	GGGGATCCTCAGCCTCTGCTGTCCTTCCG
Ca F	AACATCCAGAACCCCGACCC
Ca R	GGGGCCAGGGTTTTCCTCCAC
C140 Vα F	GCGCGGCCGCACCATGGCTCAGGAACTGGGAATG
C140 Vα R	GGGGTCGGGGTTCTGGATGTATGGGTGTACAGCCAGCCT
C140 TRBV 30 F	CGTGGAGGAAAACCCTGGCCCCATGATGCTCTGCTCTCTC CTTG
C140 TRBV 30 R	GAACACGTTCTTCAGGTCCTCTGTCACAGTGAGCCTGGTC

Table 2.2 Primers used for TCR generation by sequence overlap extension. All primers written in 5' to 3' orientation.

2.10 Table of antibodies

Antibody	source	Clone	cat num	concentration used
AmCyan conjugated anti-human CD8	BD Biosciences	SK1	339188	0.5ug
AmCyan conjugated anti-human CD3	BD Biosciences	SK7	339186	0.5ug
APC conjugated anti-human TNFa	Becton Dickinson	6401.111	340534	62.5ug
ECD conjugated anti-human CD8	Beckman Coulter	SFCI21Thy2 D3	737659	0.05ug
ECD conjugated anti-human CD4	Beckman Coulter	SFCI12T4D 11	6604727	0.025ug
APC-Cy7 conjugated anti-human CD3	BD Biosciences	SK7	557832	1ug
Pacific Blue conjugated anti-human CD3	BD Biosciences	UCHT1	558117	0.2ug
FITC conjugated anti-human CD107a	BD Biosciences	H4A3	555800	0.25ug
FITC conjugated anti-human CD4	BD Biosciences	RPA-T4	555346	0.1ug
FITC conjugated anti-human CD3	BD Pharmingen	UCHT1	555332	0.025ug
PE conjugated anti-human CD4	BD Pharmingen	RPA-T4	555347	0.025ug
PE conjugated anti-human IL2	BD Pharmingen	MQ1-17H12	559334	7.5ng
PE conjugated anti-human CD3	BD Biosciences	UCHT1	555333	0.025ug
PE conjugated anti-human Vb17	Beckman Coulter	E17.5F3.15. 13	PN-IM2048	
AmCyan conjugated anti-human CD3	BD Biosciences	SK7	339186	0.5ug
Alexa Fluor® 700 conjugated anti-human DR	BD Biosciences	G46-6	560743	0.1ug
FITC conjugated anti-human CD83	Biolegend	HB15e	305306	0.25ug
PE conjugated anti-human CD86	AbD Serotec		MCA1118PE	
PeCy5 conjugated anti-human CD40	BD Biosciences		555590	0.015ug
FITC conjugated goat anti-mouse	SIGMA	-	F2012	2ug
mouse IgG1 isotype control	R and D Systems	11711	MAB002	assay dependent
mouse IgG2b isotype control	Dako	-	MCA691	assay dependent

mouse anti EBV EA-R-p17	Millipore	5B11	MAB8188	1ng
mouse anti EBV EA-D-p52\50	Millipore	R3	MAB8186	1.6ug
mouse anti EBV ZEBRA	Santa Cruz	BZ1	sc53904	2ug
mouse anti EBNA1	AbD Serotec	O211	4260-0906	10ug
mouse anti EBNA2	Abcam	PE2	ab90543	5ug
sheep anti EBNA3a	Abcam	N/A	ab16126	1.25ug
mouse anti LMP1	Dako	CS.1-4	M 0897	4.6ug
rat anti EBV LMP2a	Santa Cruz	1579	sc101315	2ug
mouse anti EBV VCA-gp125	Millipore	N/A	MAB8184	10ug
mouse anti EBV BALF2	Kind gift of Jaap Middeldorp	OT13N	N/A	20ug

Table 2.3 Descriptions of antibodies used

CHAPTER 3

3 Selection of T-cells for TCR isolation

3.1 Target selection

3.1.1 Considerations for target selection

There are a number of important criteria to meet when selecting an antigen to target therapeutically by TCR gene transfer. Firstly, the target must be present in the tumour being treated and absent in healthy cells. This prevents on-target off-tissue toxicity. With regard to PTLD, as the majority of cases are EBV associated, here we will target an EBV derived antigen. Viral antigens may not be solely expressed on tumour cells but they will be uniquely expressed in infected cells. Therefore targeting viral antigens should result in minimal to no on-target off tumour toxicities. In the case of EBV, it is thought that other than EBV-infected malignant B-cells, only 1 in 10,000 to 1 in 50,000 circulating B-cells are infected [242]. Furthermore, as viral antigens are foreign, they are immunogenic; highly avid T-cells will not be deleted during T-cell development. TCRs with high avidity can thus be isolated

Additionally, as TCR gene transfer therapy is MHC restricted, it is advantageous to target an epitope presented through a common HLA allele so that the TCR could be used to treat many patients. Furthermore, to enable direct recognition of tumour cells, that epitope should be presented on the tumour cell surface. MHC class II complexes classically present peptides from exogenous proteins, and intercellular transfer of antigen from virus-infected cells has been demonstrated for several EBV proteins [35, 188, 189]. It is now also widely accepted that some endogenous peptides can also access the *MHC class II* pathway, and this mechanism is used to process the viral protein EBNA1 [33, 243]. Through these mechanisms,

CD4+ T-cells are able to directly target infected cells provided sufficient levels of peptide-MHC are expressed at the cell surface, and direct CD4+ T-cell recognition of LCL has now been described in the context of multiple EBV derived epitopes [189].

3.1.2 PTLD protein expression

PTLD is a heterogeneous disease; it has varied clinical presentations and histopathologies which are thought to be related to the time to PTLD onset. Due to the high degree of immune suppression, and consequent loss of T-cell control of the virus, early onset PTLD following SOT or HSCT is most often EBV associated. Here the tumours present as 'early lesions' or polymorphic lymphoproliferative disease (as defined by the WHO), and expresses an unrestricted EBV gene expression profile (latency III) [206, 207]. PTLD can also develop as late-onset (> 1 year post transplant), which is more common following SOT transplant as patients are given low level immunosuppression for life. Late-onset PTLD has been shown to express a more limited EBV gene expression profile, suggested to be caused in part by a degree of immune pressure, and as such are frequently monomorphic and often resemble HL [244]. Therefore, to successfully treat PTLD by selectively targeting specific EBV antigens, knowledge of viral gene expression is of paramount importance.

Here we have analysed the expression of multiple EBV derived proteins in 17 PTLD cases, in order to select an EBV protein to target, for the treatment of PTLD. Although some analysis of EBV protein expression in PTLD has been performed by others, many of these studies were performed using outdated techniques and included limited numbers of patients. We have collected a larger data set that includes cases of PTLD after HSCT and SOT (6 biopsies for HSCT patients and 11 biopsies from SOT patients, from the University of Birmingham Human Tissue Biorepository and The University of Nottingham Tissue Biobank) [244-250].

Additionally, we have collected data on the time between transplant and PTLD and so can compare EBV protein expression in early- and late-onset PTLD.

3.1.3 IHC and ISH of EBV derived RNA and protein

We have tested for the expression of EBERs by *in situ* hybridisation (ISH) and EBNA1, EBNA2, LMP1, BZLF1 and HLA DR at the protein level by immunohistochemistry (IHC). EBER staining was performed to determine the EBV status of the tumour. Although not all EBV positive tumour cells express EBV proteins, EBERs are constitutively expressed in all EBV positive cells [177]. This is therefore a reliable method of confirming the EBV status of the tumour. The EBER staining performed here matched with the EBER status previously determined by hospital laboratories, confirming that ISH was working as expected. EBER positivity was also confirmed by qPCR (data not shown).

The specificity of the antibodies used in IHC was demonstrated in preliminary experiments and optimised using control cells that were known to be EBV negative (tonsil samples) and EBV positive (Hodgkin's lymphoma samples) (data not shown). Controls for EBNA1, EBNA2 and LMP1 staining were cytospins of B95.8 LCLs and T-cells, which were positive and negative for the three proteins , respectively. Induced EBV positive Akata cells were used as a positive control for BZLF1 staining, as this Burkitt's lymphoma cell line can be induced by TPA into lytic cycle [251].

Following antibody optimisation, the sections from the 17 PTLD cases were stained for EBER and protein expression. Figure 3.1 shows example ISH and IHC for EBER and all proteins investigated here, on one EBV negative case and one EBV positive case. In the cases shown, strong EBER staining can be seen in the EBV positive case, whereas the EBV negative case is clearly EBV negative, suggesting that staining is specific. Additionally,

specificity is implied as non-tumour cells within the PTLD section do not stain positively for EBER. Interestingly, EBER staining across the panel of cases showed that EBV positive tumour cells in PTLD biopsies can be diffuse or clustered. Whilst the stain in Figure 3.1 shows clustered staining, many sections contained diffuse EBER positive cells.

The EBV positive case showed clear nuclear staining of EBNA1, EBNA2 and BZLF1 in the tumour cells with no staining detected in the EBV negative control case. In the case of LMP1, membrane staining was detected in the tumour cells and again the negative control case did not stain. Across the panel of cases, whilst EBNA1 appeared to be positive in 100% of tumour cells, this was not the case for EBNA2, LMP1 and BZLF1. EBNA2 is positive in the majority of tumour cells in all sections whilst LMP1 and BZLF1 are less frequently expressed. This is in line with published work in which PTLD biopsies have been stained for LMP1 and BZLF1 [245-247]. Furthermore BZLF1 is a lytic protein and as such would only be expected to be expressed in a small number of tumour cells, as only a small number of EBV infected cells undergo lytic replication at any given time [207].

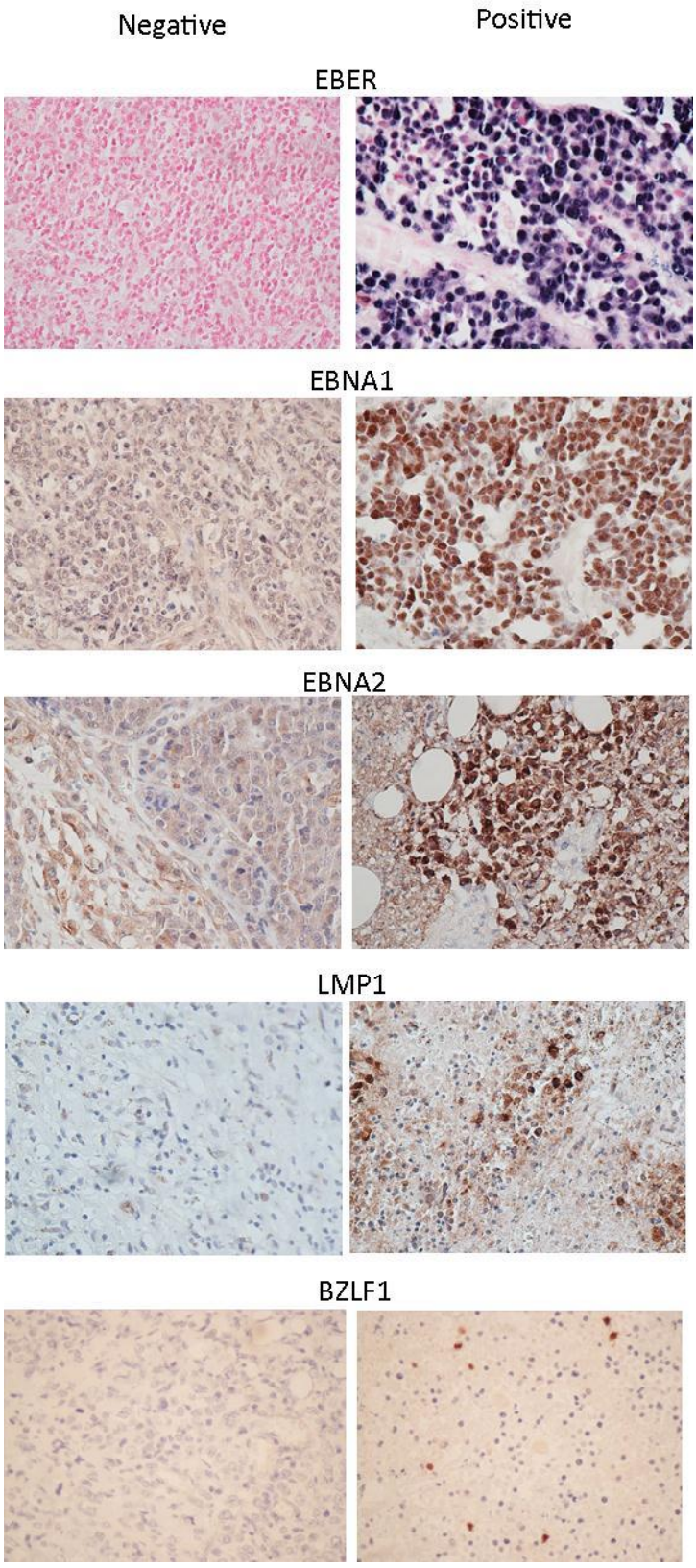


Figure 3.1 In Situ Hybridisation and Immunohistochemistry of PTLD biopsies.

Examples of EBV negative and EBV positive cases of PTLD stained by ISH for EBER and with antibodies against EBNA1, EBNA2, LMP1 and BZLF1.

	Patient	Transplant Type	Time to PTLD onset (months)	EBER	EBNA1	EBNA2	LMP1	BZ1	HLA DR
EBV Positive	1-2	HSCT	2	+	NT	+	NT	-	+
	1-3	HSCT	3	+	NT	-	+	+	NT
	A2	SOT	4	+	+	+	+	+	+
	1-5	HSCT	5	+	+	+	+	+	+
	1-6	HSCT	5	+	+	NT	+	+	+
	A	SOT	7	NT	+	+	+	+	+
	A1	SOT	7	NT	+	+	+	+	+
	4	SOT	12	+	+	+	+	+	+
	9	HSCT	12	+	+	+	+	+	NT
	C1	SOT	18	NT	+	+	+	+	NT
	5	SOT	60	+	+	+	+	+	NT
	10	SOT	96	+	+	-	+	-	NT
	11	SOT	132	+	+	-	+	NT	-
EBV Negative	1-4	HSCT	2	-	-	-	-	-	+
	A5	SOT	72	-	-	-	-	-	+
	3	SOT	96	-	-	-	-	-	NT
	2	SOT	168	-	-	-	-	-	+

Table 3.1 Summarised IHC of PTLD biopsies.

PTLD biopsies were analysed for expression of EBV genes and RNA by IHC and ISH, respectively. Gaps represent cases that were not stained for specific markers. Biopsies were analysed blind by a qualified pathologist. NT – not tested.

Table 1.1 shows a summary of the staining performed on all 17 cases, 13 EBV positive and 4 EBV negative. Results show that in line with the literature, PTLD following HSCT usually occurs within one year of transplant (early-onset PTLD) and PTLD following SOT can be early- or late-onset. EBNA1 was present in all PTLD biopsies which stained positive for EBERs. LMP1 was also present in all biopsies that were EBER positive, but in a lower percentage of cells. In the samples stained for EBNA2, the protein was present in 3/4 EBER positive HSCT samples and 6/8 EBER positive SOT samples. This suggests that 75% of samples have a latency III gene expression profile. Additionally, 4/5 and 6/8 EBV positive sections from PTLD following HSCT and SOT respectively were positive for BZLF1, suggesting that at least some cells within these tumours were able to enter lytic cycle.

3.1.4 Target Selection

Based on the expression in multiple PTLD samples as seen by IHC, we elected to targets derived from EBNA2 and BZLF1. Not only is EBNA2 expressed in PTLD, it is also expressed in AIDS- related lymphomas and a proportion (28-32%) of EBV positive DLBCL's of the elderly. As such, a treatment based on targeting EBNA2 could be used to treat multiple malignancies [252, 253]. BZLF1 is the first immediate early lytic cycle protein which is expressed in lytic cycle. It is the transcription activator which mediates the switch the lytic gene expression. Like EBNA2, BZLF1 is expressed in the majority of PTLDs and has been shown by others to be a strong candidate for T-cell targeting [254].

We selected to target the EBNA2 derived peptide, "PRS" (full sequence PRSTVFYNIPPMPLPPSQL). It has been demonstrated that the PRS epitope is processed and presented through MHC class II following antigen transfer to neighbouring cells [35].

This method of accessing the MHC class II pathway is efficient, and many antigens which access MHC class II via this route are presented at sufficiently high levels for direct CD4+ T-cell recognition [35]. Crucially, our group has shown that CD4+ T-cell clones recognised PRS naturally processed and presented by HLA DR52b at much higher levels than any other CD4+ T-cell clones recognising other epitopes. This finding is not explained by a higher functional avidity of these clones but rather the data suggest that presentation of the PRS epitope on the cell surface is more efficient with HLA DR52b [189]. Furthermore, DR52b is a common allele, expressed in 27-40% of Caucasians and so many patients could be treated with a PRS- DR52b restricted TCR [255-257]. Whilst the PRS peptide is not present in type 2 EBV due to sequence variation in EBNA2, type 1 EBV is the most common strain worldwide, and in the UK [258]. This target, presented through this HLA allele, is therefore an ideal candidate for immunotherapy.

The BZLF1 derived peptide we have chosen to target is "LTA" (full sequence LTAYHVSTAPTGSW). LTA specific CD4+ T-cells have previously been isolated and shown to respond directly to unmanipulated LCL, in both effector and cytotoxic manners, again highlighting that this peptide is presented at high enough levels on LCLs for direct CD4+ T-cell recognition. In addition, LTA is also restricted through the common MHC allele, DR52b [188]. For these reasons, we chose to begin by targeting both peptides. Whilst I had been given a PRS specific clone (clone 93) by Dr Long, we sought to generate additional PRS clones, along with LTA clones, in order to determine which had the highest therapeutic potential.

IHC has shown that not every cell within the tumour biopsy is EBNA2 or BZLF1 positive. Reasons for this are twofold. Firstly, these proteins may be present at levels below the detection limit for IHC. Secondly, some tumour cells may either not express these proteins,

or may not be expressing them at high enough levels to detect at the snapshot in time that the biopsies were taken. Brooks et al have identified cyclical expression of LMP1 in LCLs and so it is conceivable that tumour cells derived from PTLD patients could cyclically express other EBV genes [259].

Although this heterogeneity of protein expression in tumour cells could limit intercellular antigen transfer for peptide access to the MHC class II processing pathway, we hypothesise that CD4+ T-cells with redirected specificity towards these antigens would still be therapeutically useful. Firstly, tumour cells could uptake antigen in an autocrine fashion, thereby allowing access to MHC class II as exogenously derived antigen. Secondly, whilst intercellular antigen transfer is a proven route of entry into the class II processing pathway, a smaller contribution from intracellular access to this pathway, for example via autophagy, has not been ruled out. Cells that employ autophagy to present endogenously derived antigen in MHC class II do not need to be neighbouring other tumour cells and so could be directly targeted by specific CD4+ T-cells. Finally, if PRS and LTA cannot be directly presented to CD4+ T-cells on tumour cells, CD4+ T-cells could still function indirectly by helping CD8+ T-cells and stimulating a broad immune response.

Although EBNA1 and LMP1 have been identified in 100% of PTLD biopsies studied here, physiological levels of epitopes presented on the infected cell surface cannot be efficiently recognised by CD4+ T-cells. For EBNA1 this is thought to be due to the mechanism of epitope access to the MHC class II processing pathway. EBNA1 has been shown not to be transferred between neighbouring cells to enter the class II pathway, but instead is solely processed (somewhat inefficiently) by autophagy [33, 191]. LMP1 is a poor T-cell target, and no commonly recognised CD4+ T-cell epitope derived from this protein has been identified to date [166].

3.2 Isolation and characterisation of PRS- and LTA- specific CD4+ T-cells from healthy donors

3.2.1 Production of PRS-specific and LTA CD4+ T-cell clones

In order to isolate PRS and LTA specific CD4+ T-cells, we performed IFNγ capture and limiting dilution cloning on peptide-stimulated cells, as described in Materials and Methods. Briefly, we isolated PBMCs from healthy, HLA-DR52b positive, EBV seropositive donors and removed CD8+ T-cells by negative selection with dynabeads. We then exposed PBMCs to PRS and LTA peptides together to reactivate epitope specific T-cells. One week later, we re-stimulated the PBMCs with both peptides and captured cells producing IFNγ with an IFNγ secretion assay. These cells were plated out by limiting dilution cloning at 0.3, 3 and 30 cells per well into wells containing peptide-loaded autologous LCLs and PHA-treated mixed allogeneic buffy coat feeder cells (both irradiated with 4000 rads).

3.3 Characterisation of T-cell clones

3.3.1 IFNγ production in response to target peptide

The expanded T-cell cultures were screened for their specificity and functional capacity using several approaches to identify the clone with the highest therapeutic potential.

After two weeks of expansion, we screened 154 growing cultures for their specificity towards PRS or LTA. Using an IFNγ ELISA, we co-cultured the T-cells with autologous LCLs pre-exposed to PRS and LTA epitope peptides. 65 cultures produced IFNγ in response to peptide-exposed LCL and so were re-stimulated and expanded as described. The remaining cultures did not secrete IFNγ in response to peptide exposed LCLs and so were deemed non-specific or exhausted T-cells and discarded.

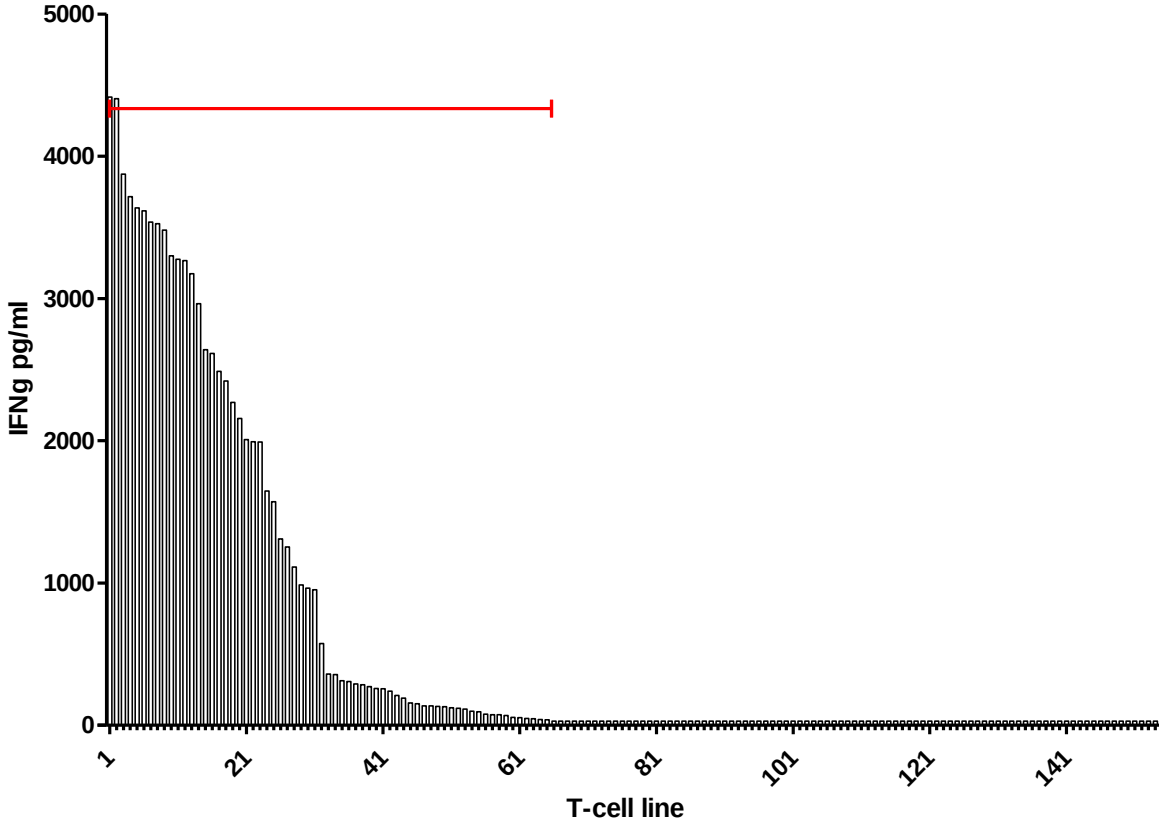


Figure 3.2 IFNγ production in response to LTA- and PRS-exposed LCL.

LCL was exposed to LTA or PRS epitope peptide and then co-cultured with T-cells for 16 hours, before supernatant was analysed in an IFNγ ELISA. Background responses to LCL pre-incubated with the DMSO solvent have been subtracted. Red line indicates the clones picked for further analysis.

Responding cell lines that continued to grow well in culture were then tested a second time using the same assay to determine whether the cells were PRS or LTA specific. Here, we stimulated all T-cell cultures with PRS and LTA peptides separately. Results show that of the 48 cultures screened, 30 produced IFNγ at high levels to either PRS or LTA (Figure 3.3). Of these, 24 were carried forward for further analysis. Cell lines that produced IFNγ in response to targets but were not carried forward did not continue to grow, suggesting the cell line may have been exhausted.

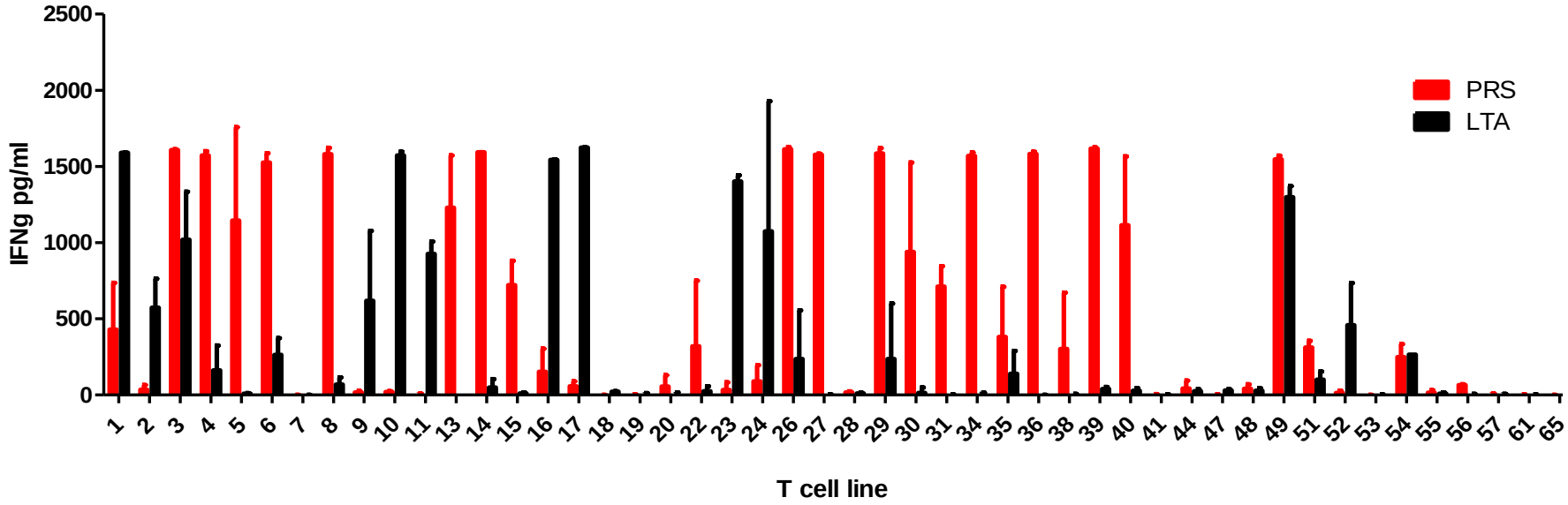


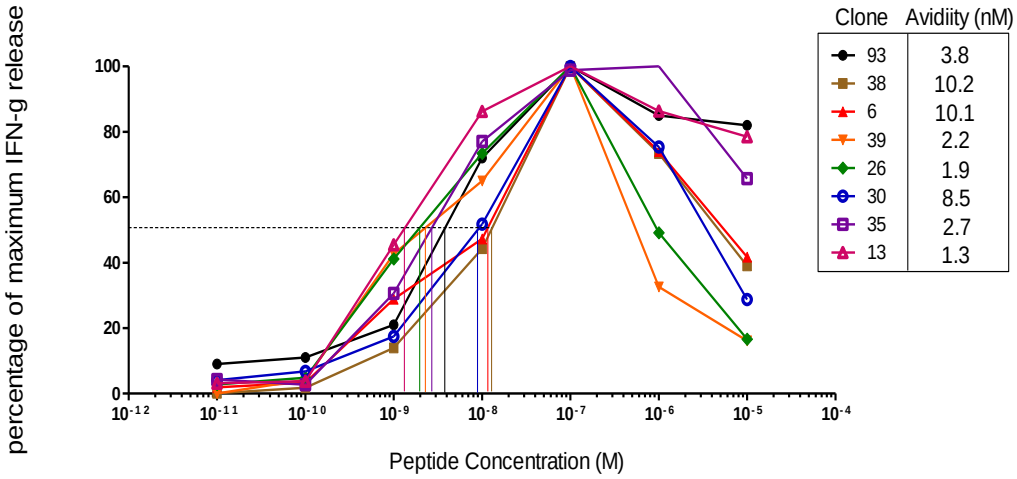
Figure 3.3 IFNγ production in response to PRS or LTA.

Selected T-cell cultures were analysed for specificity against LCLs pre-exposed to either PRS or LTA by IFNγ ELISA after 16 hours co-culture. Background responses of T-cells cultured with DMSO have been subtracted.

3.3.2 Determination of T-cell functional avidities

To determine which T-cell cultures showed highest avidities for the PRS or LTA peptides, we determined T-cell functional avidity using a peptide titration assay, again measuring IFNγ release by ELISAs (Figure 3.4). Here, I compared the cell lines I had generated to a clone (clone 93) previously generated in the same manner by Dr. H. Long [189]. To remove complication by background recognition of the unmanipulated target lines, these assays used LCLs transformed with EBV lacking the relevant epitope sequences. Thus, PRS specific cultures were co-cultured with Ag876 LCLs, either pre-exposed to epitope peptide or not. Ag876 is an EBV type 2 strain that carries an EBNA2 gene with a number of sequence changes from the standard type 1 EBV stain, B95.8 [260]. Consequently, the PRS peptide sequence differs to the sequence present in B95.8 EBV, and as such ablates PRS-specific T-cell recognition. LTA specific T-cell lines were co-cultured with LCLs which have previously been genetically engineered to prevent them from expressing the BZLF1 protein (BZLF1 knock out (K/O) LCL). As BZLF1 K/O LCLs do not express BZLF1, they do not present LTA unless loaded exogenously with the epitope peptide [261]. In these experiments, we defined functional avidity as the peptide concentration required to produce half the maximal response. The results in Figure 3.4 show that we have successfully isolated T-cell cultures specific for either LTA or PRS with a range of avidities from 1.3 to 19nM. This shows that the T-cell lines generated here have high avidities in comparison to other EBV specific CD4+ T-cells within the literature [189].

A) PRS-specific clones



B) LTA-specific clones

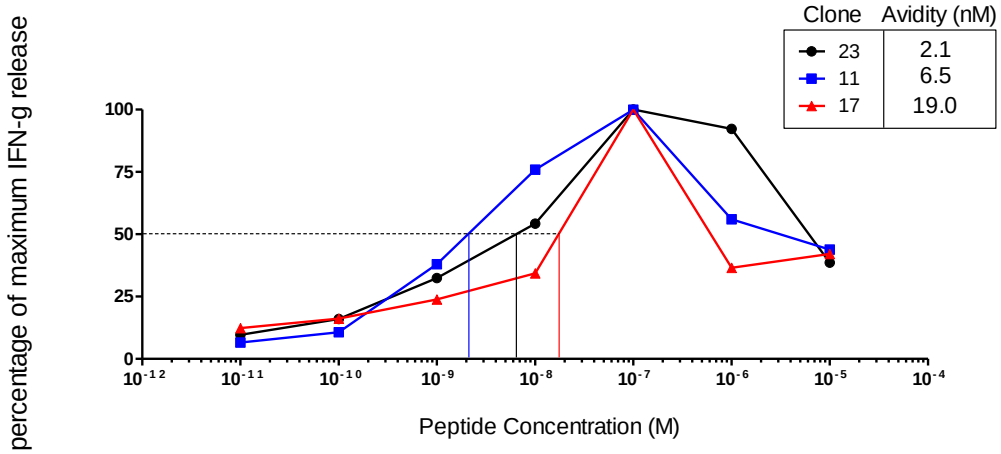


Figure 3.4 Peptide titrations of T-cell cultures.

A) T-cell cultures that previously responded to PRS peptide were co-cultured for 16 hours with autologous Ag876 LCL pre-exposed to various concentrations of PRS peptide. B) T-cell cultures that previously responded to LTA peptide were co-cultured with LTA-exposed BZLF1 knock out LCL for 16 hours…as above. Both culture supernatants were then analysed by IFNγ ELISA.

3.3.3 Confirmation of T-cell HLA restrictions

The HLA restriction of isolated T-cell cultures is important therapeutically, as we can only treat patients who share the same HLA allele that the T-cell is restricted by. Therefore, the isolated T-cells should ideally be restricted through common HLA antigens. Previously published work has identified that PRS is restricted through multiple HLA alleles, and can be presented in the context of DR1, DR7, DR16, DQ2, DQ7, DR52a, DR52b and DR52c [189, 262]. LTA has been described previously as being DR52b restricted [188].

We aimed to isolate DR52b restricted TCRs, as this HLA allele is frequent in the population. We therefore isolated PBMCs from healthy donors who express DR52b and do not express other HLA alleles known to present PRS. As such, we hypothesised that the T-cell cultures we had isolated would be restricted through this HLA allele. We validated the HLA restrictions of promising CD4+ T-cell cultures using a panel of LCLs which included the autologous LCL and a range of lines that were partially matched with the autologous donor at different class II alleles. Figure 3.5 shows the HLA restriction of the LCLs used. If the T-cells were restricted through DR52b, they should respond to donors one, two and three which share the DR52b allele, and not produce IFNγ in response to donors four and five, which do not express DR52b. All T-cell cultures tested produced IFNγ when co-cultured with DR52b+ LCLs. However, clone 23 and 13 produced some IFNγ in response to DR52b- LCLs, suggesting a possibility of oligoclonal T-cell cultures. For the other clones tested, a DR52b restriction was evident.

A)

Donor	**HLA type**					
	DR				DQ	
1	11	13	52a	52b	6	7
2	11			52b	6	7
3		13		52b	6	
4			52a		6	
5						7

*Donor 1 is autologous. Donors 2-5 are partially HLA matched and share some of the same class II HLA alleles as donor 1.

B)

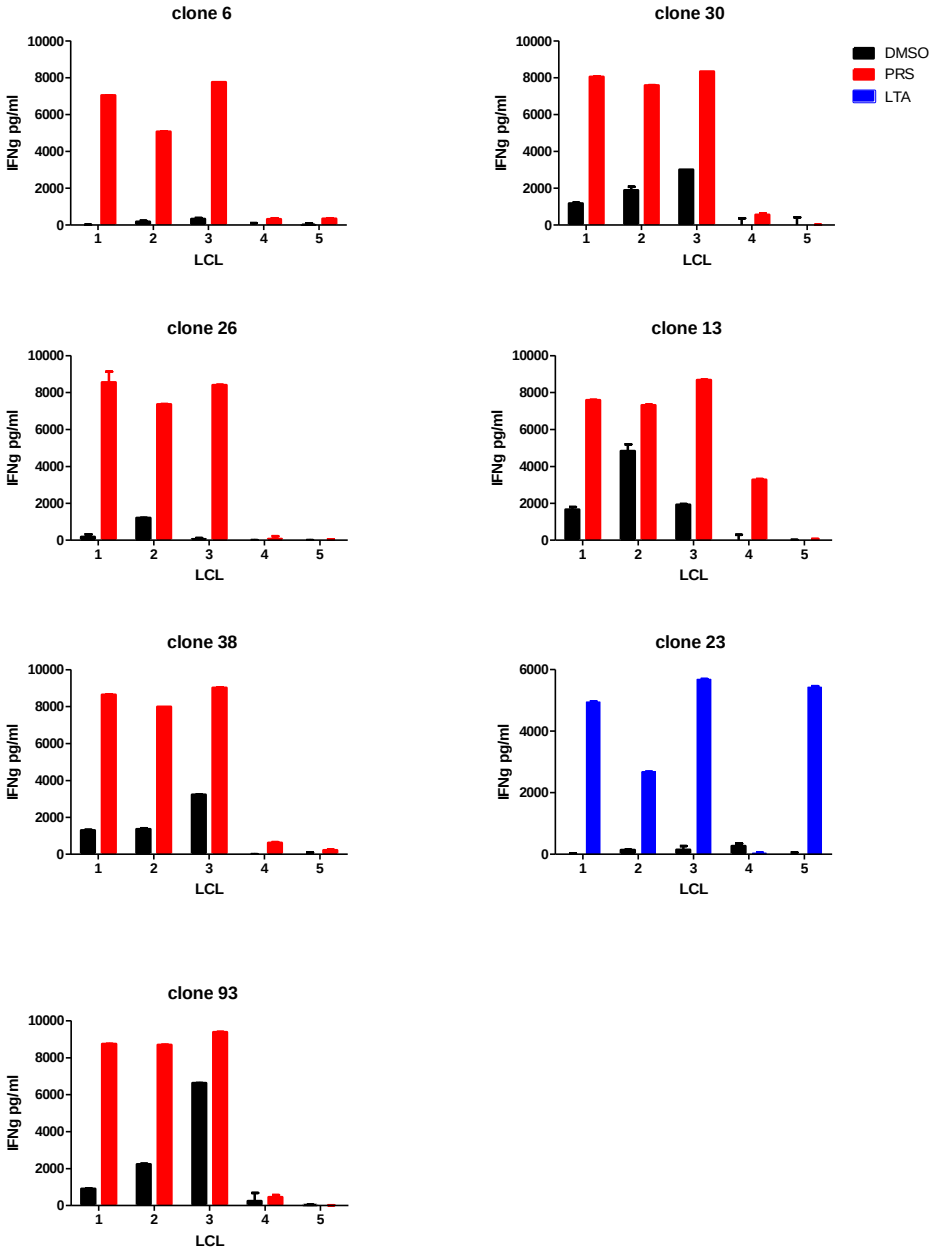


Figure 3.5 HLA restrictions of T-cells.

$5x10^4$ T-cells/well were co-cultured with $1x10^5$ LCLs of defined HLA type which had or had not been pre-exposed to epitope peptide. After 16 hours co-culture, supernatants were analysed for T-cell response by IFNγ ELISA. IFNγ produced by LCLs and T-cells alone was subtracted from results shown, although these levels were consistently low (below 1% of the amounts produced by co-cultures).

3.3.4 T-cell responses to naturally processed and presented target epitope

The T-cell response to peptide-exposed targets is important as it confirms that the T-cell cultures isolated are capable of responding to their cognate antigen, and also determines their avidity. However, for these T-cell responses to be therapeutically relevant they must be capable of responding to levels of antigen that are naturally presented on the infected cell's surface. To explore the ability of our T-cell cultures to respond to naturally processed and presented antigen we calculated the 'efficacy' of T-cell responses to LCL.

The efficacy of the T-cell cultures was analysed by co-culturing the T-cells with DR52b positive LCL (carrying the B95.8 EBV strain) pre-exposed to the target peptide epitope (or to DMSO solvent only as a control), and DR52b positive Ag876 LCLs and BZLF K/O LCLs. DR52b negative B95.8 LCLs were also used as a further negative control. The efficacy with which the T-cell cultures recognised unmanipulated LCL was expressed as a percentage of maximal response seen against the same targets loaded with an optimal epitope peptide concentration (Figure 3.6).

Efficacy was calculated as:

$$\frac{\text{pg/ml of IFN}\gamma \text{ produced by T-cells in response to LCL}}{\text{pg/ml of IFN}\gamma \text{ produced by T-cells in response to LCL pre-exposed to peptide.}} \times 100$$

Figure 3.6 shows three representative T-cell efficacy graphs. There was a range of efficacies amongst the T-cell lines tested and 7 cell lines tested had efficacies over 10%. There was no correlation between T-cell avidity and percentage efficacy of LCL recognition (correlation

efficient 0.09), indicating that at least for these cell lines, levels of peptide:MHC naturally presented on EBV-infected B cells were sufficient to fully stimulate the T-cells for IFNγ release regardless of the avidity of the T-cell culture. Clone 93 (c93) has previously been shown to respond to unmanipulated LCL well, producing 35% of the IFNγ that it produces in response to peptide exposed LCL (35% efficacy) [189].

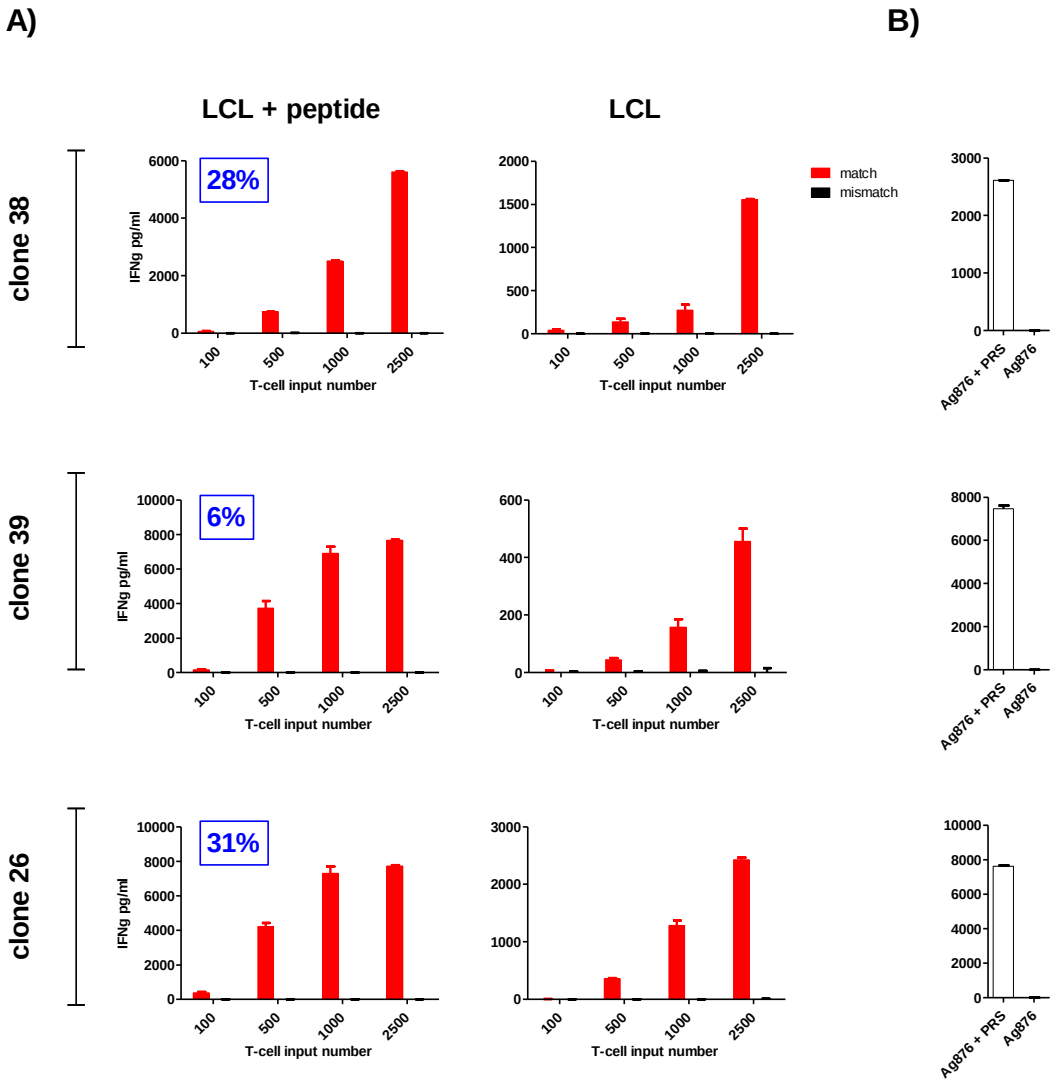


Figure 3.6 T-cell efficacy.

T-cells were co-cultured with targets for 16 hours and supernatants analysed by IFNγ ELISA. A) T-cell response using different T-cell input numbers to LCLs with or without peptide-loading B) T-cell response to Ag876 LCL with 2500 T-cells/well. In all cases IFNγ release levels from T-cells alone have been subtracted. Blue boxes show efficacy of responding cells.

3.3.5 T-cell ability to bind soluble peptide-MHC

To further investigate the specificity of our T-cell cultures at a single cell level, we stained the T-cells with soluble PRS-DR52b molecules which had been linked by streptavidin to generate tetramers and PE-conjugated to allow for identification (PRS-DR52b tetramers). Unlike IFNγ ELISAs, using p-MHC tetramers within flow cytometry provides information on the proportion of cells within the population that carry the correct TCR to bind to a given p-MHC complex. Results showed that in T-cell lines 26, 13, 38 and 93, between 92.9% and 98.7% of cells bound the tetramer, suggesting that they may be monoclonal populations. T-cell line 6 is positive for tetramer staining; however two peaks suggests at least two separate PRS-specific T-cell clones with different abilities to bind the tetramer. This could be due to different levels of TCR expressed at the cell surface or different abilities of the TCRs present to bind tetramer. T-cell lines 30 and 23 show clear oligoclonal populations, as 73% of T-cell line 30 and 42% of T-cell line 23 bind to the tetramer.

That only 42% of T-cells in line 23 express the DR52b restricted LTA TCR backs up the suggested oligoclonality observed in the HLA restriction assay (Figure 3.7). Here, the T-cell line responded to LCL 5, which did not express DR52b, along with all LCLs that did express DR52b (LCLs 1, 2 and 3). With a T-cell population where less than half of the cells express the desired TCR, cloning the correct TCR alpha and beta genes would prove difficult. As a consequence, this cell line was not investigated further. As clone 23 was the only LTA specific clone to survive and expand *in vitro* to sufficient levels for functional analysis, LTA specific TCR gene transfer was dropped at this point and following work was focused on PRS-specific TCRs.

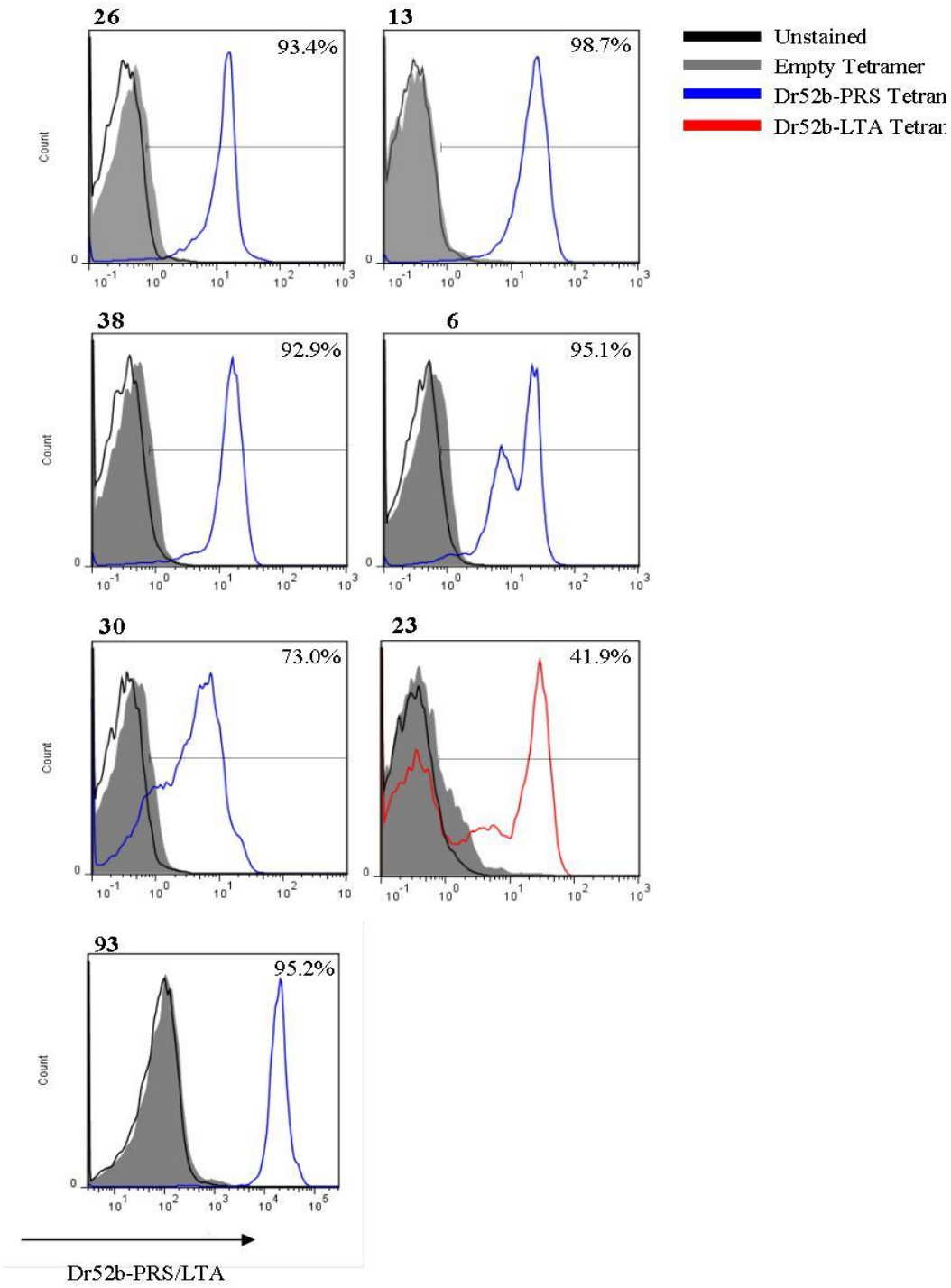


Figure 3.7 Tetramer staining of T-cell cultures.

T-cells were stained with PE-conjugated LTA- or PRS-DR52b tetramers, or empty tetramer negative controls. Cells were gated on lymphocytes then live cells.

3.3.6 Identification of T-cell variable beta genes

Successful cloning of the required TCR genes is made much easier if the T-cell population is monoclonal, to reduce the chance of isolation of non-specific TCR genes from contaminating T-cells. Therefore, before attempting to clone the alpha and beta TCR genes, we identified which variable beta chains were expressed in the T-cell cultures. This allowed us to look further into the clonality of the T-cell lines.

To determine which beta chains the T-cell cultures expressed we extracted cellular RNA, performed reverse transcription to generate cDNA and then conducted PCR reactions using a panel of primers specific for different V-beta alleles (primer sequences are described in materials and methods Table 2.1). Each T-cell can express one variable beta allele. There are 24 variable beta families, with an estimated 57 variable beta alleles [263]. Our primer panel allowed us to test for 24 alleles. As such, this panel will not conclusively determine T-cell clonality, as there could be T-cells in the culture containing an allele that is not tested for here. Alternatively, two different T-cell clones in one culture may share the same beta allele. Nevertheless, in combination with tetramer staining, we can increase our chances of identifying a monoclonal T-cell population.

As can be seen in Figure 3.8, cell lines 13 and 6 are positive for multiple Vβs and therefore were rejected.

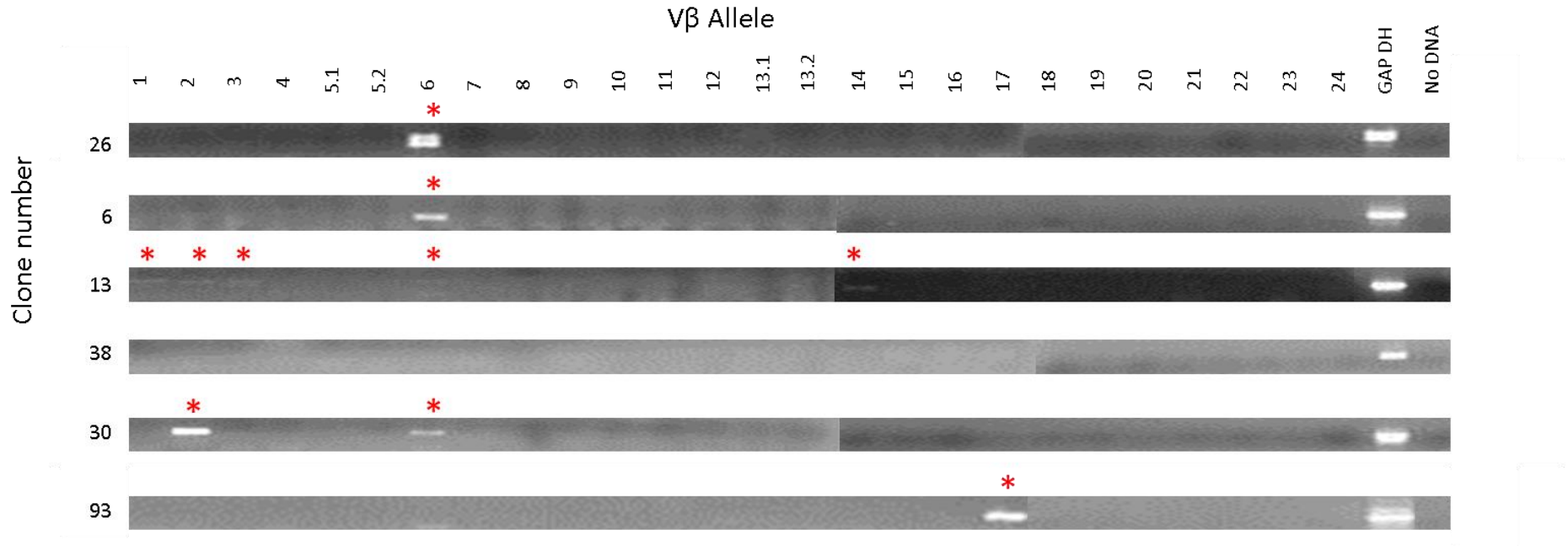


Figure 3.8 PCR analysis of Vβ chain usage in T-cell clones.

Shown regions are 200 to 300 base pairs (expected beta fragment size) and 500 base pairs (GAPDH size). Red asterix highlights where bands were observed.

3.4 Summary of cloning and analysis

From the collated T-cell analysis performed here, we decided to clone the T-cell receptor from c93. This clone appears monoclonal by Vβand tetramer analysis, has a high avidity and is restricted through the correct HLA allele. Importantly for therapy, it is also able to recognise and respond to LCLs presenting physiological levels of peptide, as is evident from it's efficacy of 35%.

Clone	T-cell avidity (nM)	Functional Efficacy	DR52b restricted	Tetramer staining DR52b-epitope MFI	Vβ PCR bands
26	1.9	31%	Yes	Single peak	1
13	1.3	45%	Yes	Single peak	5
38	10.2	27%	Yes	Single peak	0
6	10.1	6%	Yes	Two peaks	1
30	8.5	6%	Yes	Two peaks	2
23	2.1	27%	Yes	Two peaks	N/A
93	3.8	35%	Yes	Single peak	1

Table 3.2 Summary of results from T-cell clones with therapeutic potential.

3.5 T-cell Receptor Cloning

To isolate the alpha and beta genes from c93, we used the Clontech SMART RACE cDNA Amplification Kit according to the manufacturer's instructions. We first converted poly A+ RNA into cDNA, and then amplified both the □ and □ TCR genes using Rapid Amplification of cDNA Ends (RACE) PCR with specific alpha and beta primers (see materials and methods).

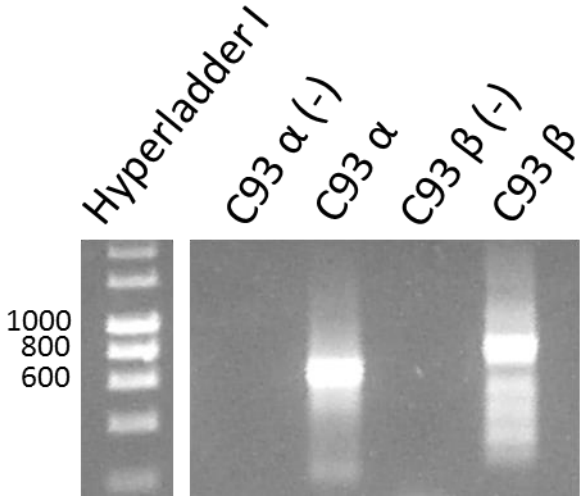


Figure 3.9 c93 alpha and beta TCR chain PCR.

Alpha and beta TCRs were amplified using alpha and beta gene specific primers and a universal primer mix. C93 α (-) and C93 β (-) are negative controls which do not contain DNA.

Resulting DNA was run on an agarose gel, the appropriate bands extracted, and amplified through bacterial transformation. Alpha and beta TCR chains were then sequenced. The variable alpha allele used in this TCR was TRAV5*01 (IMGT nomenclature), also known as Vα15.1 (Wei et al. nomenclature; used throughout this thesis) [264]. The joining region allele

used in this alpha TCR chain was TRAJ8*01. The beta chain used the TRBV19*01 variable allele (Vβ17) and the TRBJ1-1*01 joining allele.

The isolated TCR alpha and beta chains were ligated into a pMP71 retroviral plasmid (kindly provided by C. Baum, Hannover, Germany). The plasmid that was used for these experiments had TCR genes already cloned into it and these genes and been codon optimised (by GeneArt) to enhance the efficacy of translation. The alpha and beta chains were separated by a 2A peptide linker from porcine Teschovirus. This ensured equimolar expression of both genes as at translation the ribosome 'skips' the 2A linker, preventing peptide bond formation and consequently cleaving the polypeptide chain [265]. Furthermore, the constant chains had previously been adapted to contain two extra cysteine residues which generate an additional disulphide bond to aid correct TCR chain pairing in transduced cells [235].

In order to insert alpha and beta variable, joining and diversity sequences into this plasmid (thereby replacing the existing variable, joining and diversity regions but retaining the codon optimised constant domains), primers were designed to extend the alpha and beta sequences of the TCR genes from c93 with correct nucleotides to allow for insertion (see Figure 3.10). The alpha chain was extended to include a 5' Not1 restriction enzyme site followed by a Kozak sequence (CCACCATGG). The 3' end of the alpha chain was extended to include a constant chain overlap to allow for PCR driven joining of alpha variable and joining regions to codon optimised constant domain. The beta chain was extended to include a 5' overlap to the p2A linker and 3'overlap to allow for PCR driven joining of the beta variable joining and diversity regions to the beta constant domain. Finally, the beta constant domain was extended with a 3' stop codon and BamH1 restriction site.

After performing the multiple PCRs required to construct the TCR alpha-p2A linker-beta fragment, we inserted this into an MP71 retroviral vector by restriction enzyme digestion of

the parental MP71 vector to remove the original TCR, and subsequent ligation of the newly amplified TCR gene fragments. After amplification of the plasmid in bacteria and sequence confirmation of the new PRS-TCR retroviral vector, we proceeded to transduce and analyse healthy donor T-cells as described in Chapter 4.

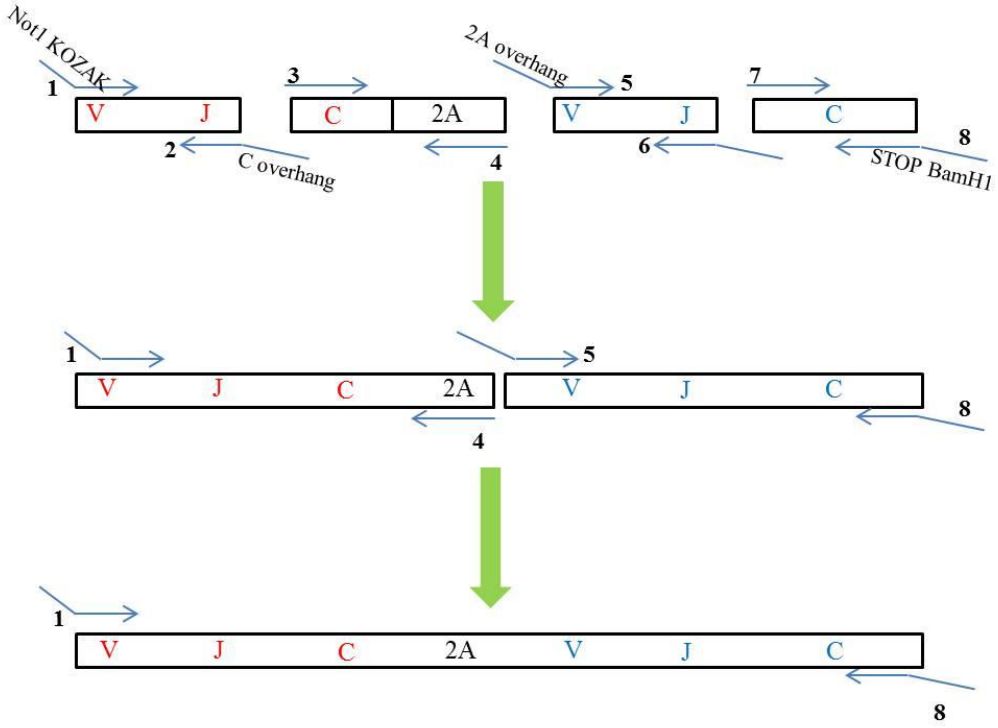


Figure 3.10 TCR cloning strategy

Primer combinations 1 and 2, 3 and 4, 5 and 6 and 7 and 8 were used to generate the first fragments. The first two DNA products were then connected using primers [1 and 4] and the latter two using primers [5 and 8]. To get a final, linear DNA fragment, primers [1 and 8] were used. Primer 1 contained the Kozak sequence CCACCATGG and primer 8 contained the stop codon TGA.

Sequencing of the construct confirmed that we had successfully cloned the correct TCR into the vector (see Figure 3.11).

ATGGAGACATTTGCTGGATTTTCGTTCCTGTTTTTGTGGCTGCAGCTGGACTGTATGAGTAGAGGAGAGGATG
TGGAGCAGAGTCTTTTCCTGAGTGTCCGAGAGGGAGACAGCTCCGTTATAAACTGCACTTACACAGACAGCTC
CTCCACCTACTTATACTGGTATAAGCAAGAACCTGGAGCAGGTCTCCAGTTGCTGACGTATATTTTTTCAAATA
TGGACATGAAACAAGACCAAAGACTCACTGTTCTATTGAATAAAAAGGATAAACATCTGTCTCTGCGCATTGC
AGACACCCAGACTGGGGACTCAGCTATCTACTTCTGTGCAGAGAAGGGAAGGAACACAGGCTTTCAGAAACT
TGTATTTGGAACTGGCACCCGACTTCTGGTCAGTCCAAACATCCAGAACCCCGACCCCGCCGTGTACCAGCTG
CGGGACAGCAAGAGCAGCGACAAGAGCGTGTGCCTGTTCACCGACTTCGACTCCCAGACCAACGTGTCCCAG
AGCAAGGACTCCGACGTGTACATCACCGACAAGTGCGTGCTGGACATGCGGAGCATGGACTTCAAGAGCAAC
AGCGCCGTGGCCTGGTCCAACAAGAGCGACTTCGCCTGCGCCAACGCCTTCAACAACAGCATCATCCCCGAGG
ACACCTTTTTCCCCAGCCCCGAGAGCAGCTGCGACGTGAAACTGGTGGAGAAGTCCTTCGAGACAGACACCA
ACCTGAACTTCCAGAACCTGAGCGTGATCGGCTTCAGAATCCTGCTGCTGAAAGTGGCTGGATTCAACCTGCT
GATGACCCTGCGGCTGTGGAGCAGCGGCAGCGGCGCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACG
TGGAGGAAAACCCTGGCCCCATGGGCAACCAGGTGCTCTGCTGTGTGGTCCTTTGTCTCCTGGGAGCAAACAC
CGTGGATGGTGGAATCACTCAGTCCCCAAAGTACCTGTTCAGAAAGGAAGGACAGAATGTGACCCTGAGTTG
TGAACAGAATTTGAACCACGATGCCATGTACTGGTACCGACAGGACCCAGGGCAAGGGCTGAGATTGATCTA
CTACTCACAGATAGTAAATGACTTTCAGAAAGGAGATATAGCTGAAGGGTACAGCGTCTCTCGGGAGAAGAA
GGAATCCTTTCCTCTCACTGTGACATCGGCCCAAAAGAACCCGACAGCTTTCTATCTCTGTGCCAGTACCCCTCC
CGGGACAGAGAAAGCTGAAGCTTTCTTTGGACAAGGCACCAGACTCACAGTTGTAGAGGACCTGAAGAACGT
GTTCCCCCCCGAGGTGGCCGTGTTCGAGCCCAGCGAGGCCGAGATCAGCCACACCCAGAAAGCCACCCTGGT
GTGCCTGGCCACCGGCTTCTACCCCGATCACGTGGAGCTGTCTTGGTGGGTGAACGGCAAAGAGGTGCACTC
CGGCGTCTGCACCGACCCTCAGCCCCTGAAAGAGCAGCCCGCCCTGAACGACAGCCGGTACTGCCTGTCCTCC
CGGCTGAGAGTGTCTGCTACATTCTGGCAGAATCCCCGGAACCACTTCCGGTGCCAGGTGCAGTTCTACGGCC
TGAGCGAGAACGACGAGTGGACCCAGGACAGAGCCAAGCCCGTGACCCAGATCGTGTCCGCCGAGGCCTGG
GGCAGAGCCGACTGCGGCTTCACCAGCGAGAGCTACCAGCAGGGCGTGCTGTCTGCCACCATCCTGTACGAG
ATCCTGCTGGGCAAGGCCACCCTGTACGCCGTGCTGGTGTCCGCCCTGGTGCTGATGGCCATGGTGAAGCGG
AAGGACAGCAGAGGCTGA

Figure 3.11 Sequence of clone 93 TCR in pMP71 vector. Red font indicated variable regions. Purple font indicates joining regions. Blue font indicates constant regions. Yellow font indicates the GSG spacer and green font indicates the linker sequence.

3.6 Discussion

3.6.1 Overview

Here, we have isolated and cloned CD4+ T-cells from healthy donors against the PRS and LTA epitopes, derived from the proteins EBNA2 and BZLF1, respectively. Both EBNA2 and BZLF1 are expressed in multiple EBV associated malignancies including PTLD, AIDs related lymphoma and DLCBL [247, 266-271].We hypothesise that CD4+ T-cells that can recognise and respond to physiological levels of cognate antigen presented on the surface of EBV-infected B cells have therapeutic potential. Furthermore, such T-cells that directly interact with the target T-cell should have both direct effector and helper functions [188,

189]. As anti tumour responses are enhanced in the presence of both effector and helper activity, this could further increase their therapeutic efficacy [272].

3.6.2 Target Selection

To select an EBV derived antigen to target, we analysed the expression of multiple EBV proteins on PTLD biopsies from 17 patients. The panel included 6 biopsies from patients following HSCT and 11 biopsies from patients following SOT. We showed that EBNA1 and LMP1 were expressed in 100% of EBV positive biopsies and EBNA2 and BZLF1 were expressed in 75% and 77% of EBV positive biopsies, respectively. Whilst EBNA2 and BZLF1 were expressed less frequently that EBNA1 and LMP1, we selected to target antigens derived from these proteins. Reasons for this include that epitopes derived from EBNA1 and LMP1 are not presented at high enough frequencies on the surface of target T-cells in MHC class II complexes for direct CD4+ T-cell recognition. However, EBNA2 and BZLF1 are both well-presented and it has previously been demonstrated that T-cells that target antigens from these proteins can respond directly to physiological levels of antigen, by producing cytokines and cytotoxic responses [188, 189]. As the frequency of PTLDs positive for these two proteins is high, and the antigens are presented by common MHC alleles, a significant number patients would be able to benefit from TCR gene therapy against these epitopes.

3.6.3 TCR selection

One hundred and fifty four expanded T-cell cultures were sequentially subjected to a range of assays in order to determine which has the most potential to be used therapeutically. Selection was based on several assays that allowed us to investigate a range of T-cell functions. These included identifying PRS- and LTA- specific cultures that displayed high

avidity for their target antigen, identifying clones that were DR52b-restricted, and those that efficiently recognised EBV-infected B cells (a model for PTLD), and appear monoclonal.

The functional readout used for much of this screening was IFNγ production. IFNγ has been shown to have a multitude of immunostimulatory effects, and therefore T-cells that produce IFNγ are considered clinically beneficial for cancer therapy. Moreover, IFNγ is a cytokine produced by Th1 and Th17 CD4+ T-cells, both of which have been shown to have anti-tumour effects, and are capable of recruiting and promoting a larger immune response [273]. Nevertheless, this approach will have missed specific T-cells that responded to their target but without the release of this particular cytokine. Alternative selection of tetramer stained T-cells can avoid this problem but does not provide a reliable functional readout to determine avidity.

3.6.4 T-cell specificity

We first performed peptide recognition studies to allow us to distinguish between cell cultures which responded to the target peptides and non-peptide-specific cell cultures that were captured by the IFN capture assay.

Of 154 growing cultures, 65 cell lines produced peptide in response to peptide loaded LCLs. As all the cells were isolated using an IFNγ secretion assay, all isolated cells should produce IFNγ in response to peptide. The lack of IFNγ production by 89 of the 154 cultures may have been due to ‘background selection’ of non-responding T-cells, or isolation of cells that secreted IFNγ due to bystander activation. Alternatively, PRS and LTA specific effector T-cells that produced IFNγ in the initial selection assay may have been highly differentiated effector cells that became exhausted after repeated stimulations [274]. This would have rendered the specific T-cells unable to produce cytokines and would have resulted in non-

specific T-cells within the culture overgrowing the exhausted T-cells, which would not have proliferated.

3.6.5 T-cell functional avidities

The functional avidities of the epitope-specific T-cells seen here are high in comparison with those reported for other CD4+ T-cell clones in the literature. The T-cells studies here have avidities ranging between 1.3 and 19nM. Long et al. have investigated the avidities of a range of EBV specific CD4+ T-cell clones and report avidities ranging from 15-100nM, indicating that the T-cell avidities seen here are comparable to those of other viral specific TCRs [189]. Other EBV specific TCRs have been shown to have a high functional avidity, with a class I restricted TCR against an A2-restricted LMP2-derived peptide (CLGGLLTMV) having a functional avidity of 1nM when transduced into CD8+ T-cells and 10nM when transduced into CD4+ T-cells [237]. Interestingly, a number of CD8+ T-cell functional avidities against non-EBV viruses have been published, and a large range of avidities are noted, from 10pM to 1μM [275-277]. Generally, T-cells specific to antigens overexpressed in cancers, such as the cancer testis antigen MART1 have much lower functional avidities than T-cells specific for virus derived antigens. Clinically tested T-cells that are specific to MART1 have a functional avidity of 0.1μM [278].

High affinity TCRs are beneficial therapeutically, as the target T-cell could be recognised when expressing lower levels of antigen-MHC compared to what would be required for recognition by low affinity TCRs. Indeed, Adaptimmune, Oxford, UK, who focus on TCR gene transfer, use affinity maturation to enhance the affinity of tumour specific TCRs, highlighting the importance of this T-cell parameter.

Continuing this work with a high avidity T-cell could therefore show benefit *in vivo*. To further characterise our T-cell clones, we tested for their efficacy at recognising and responding to EBV-infected cells expressing physiological levels of their target antigens.

3.6.6 T-cell response to physiological levels of target epitope

We determined the efficacy of our cloned T-cells by calculating to what extent they were able to respond to LCL, in comparison to their maximal response (determined using LCLs exposed to an optimal concentration of peptide). As LCLs are EBV infected B-cells expressing a latency III profile they are a good model for PTLD, naturally processing and presenting the same antigens. Due to this similar gene expression, T-cells that are able to respond to naturally presented peptides on LCLs through HLA class II alleles have the best chance of therapeutic success for PTLD patients. Furthermore, T-cells that can recognise EBNA2 on LCLs could be therapeutically useful for the treatment of some AIDs related lymphomas and DLBCLs which express this EBV latent protein. Of the T-cell lines analysed here, 7 cell lines responded to physiological levels of antigen with efficiencies over 10%. Previous analysis of CD4+ T-cell clone efficacies towards EBV latent protein derived epitopes have identified 3 clones that could recognise more than 10% of the maximal IFNγ response (an $EBNA2_{11\text{-}30}$ GQT specific clone recognised unmanipulated LCL with an efficacy of 15%, a different $EBNA2_{276\text{-}295}$ PRS specific clone had a 15% efficacy and the clone used here showed an efficacy of 35%). This shows that we have isolated a range of T-cell lines which could potentially be used to treat EBNA2 expressing malignancies.

3.6.7 T-cell clonality

Having selected T-cell lines based on these functional tests, we moved on to check T-cell clonality. Clonality is important, as having a pure T-cell population of high avidity allows us

to clone a T-cell receptor with ensured high avidity. An oligoclonal population may result in cloning a TCR with a lower avidity, or alpha and beta chains from different clones.

3.6.8 Final TCR selection

Following all the screening tests, we selected clone (PRS-specific) for TCR cloning because it showed high avidity, restriction through the desired HLA allele and a good response to unmanipulated LCL. We isolated the TCR encoding genes from this clone, inserted them into a retroviral expression vector optimised for TCR transduction, and sequenced the construct to confirm correct TCR gene insertion.

CHAPTER 4

4 *In vitro* functionality of PRS-TCR transduced T-cells

With the intention of investigating the therapeutic potential of EBV-specific, class II-restricted TCRs, I have isolated and cloned a high avidity TCR that recognises the EBNA2 derived epitope PRS in the context of HLA-DR52b, as described in chapter 3. We hypothesise that this TCR will be useful in the treatment of EBV-associated tumours which express EBNA2. These include PTLD, AIDs related lymphoma and DLBCL. Here, we proceeded to express the isolated TCR in PBMCs derived from healthy subjects and investigate it's functionality through a range of *in vitro* assays.

The direct recognition and cytotoxicity of EBV-specific CD4+ T-cell clones against EBV-infected LCLs expressing physiological levels of their target antigens has previously been demonstrated [188, 189]. We have isolated a TCR against PRS, as this epitope is presented at high enough levels on the surface of LCLs to allow for direct CD4+ T-cell-mediated recognition and cytotoxicity. We therefore hypothesised that transduced T-cells would be similarly able to respond in an effector and helper manner *in vitro*, suggesting therapeutic potential *in vivo*. We have investigated the response of PRS-TCR transduced T-cells through a range of *in vitro* functional studies.

4.1 Surface expression of exogenous TCR

T-cells from healthy donors were activated *in vitro* with 30ng/ml Okt3, 30ng/ml anti-CD28 and 600U/ml IL2 and transduced with a MP71 retroviral vector encoding the PRS-specific TCR from c93, as described in materials and methods. In order to function, the TCR genes

must be inserted into the genome, transcribed and translated correctly, and trafficked to the cell surface. In order to confirm surface expression of the introduced TCR and determine the efficacy of transduction, transduced cells were stained with an antibody against the specific TCR V beta variable chain and with a specific peptide-MHCII tetramer.

As all T-cells express a native TCR prior to transduction, the introduced TCR is referred to as exogenous and the original TCR as endogenous. We had previously determined, by both PCR and sequencing, that the β-chain of our PRS-specific TCR is Vβ17 (chapter 3). Herein, we therefore analysed the efficiency of every transduction using surface staining for Vβ17. Interestingly, both CD4+ and CD8+ T-cells were effectively transduced (Figure 4.1). In all transductions performed, there was a small level of Vβ17 staining in non-transduced (mock) populations. This represents the frequency of naturally occurring Vβ17 usage in the host's TCR repertoire. Whilst different donors have been used throughout this work as a source of PBMCs, in each experiment we generated a control mock-transduced cell line from the same donor. Therefore, we were able to determine the percentage of engineered Vβ17+ T-cells within the transduced T-cell line by subtracting the percentage of naturally occurring Vβ17+ cells present in the mock-transduced line.

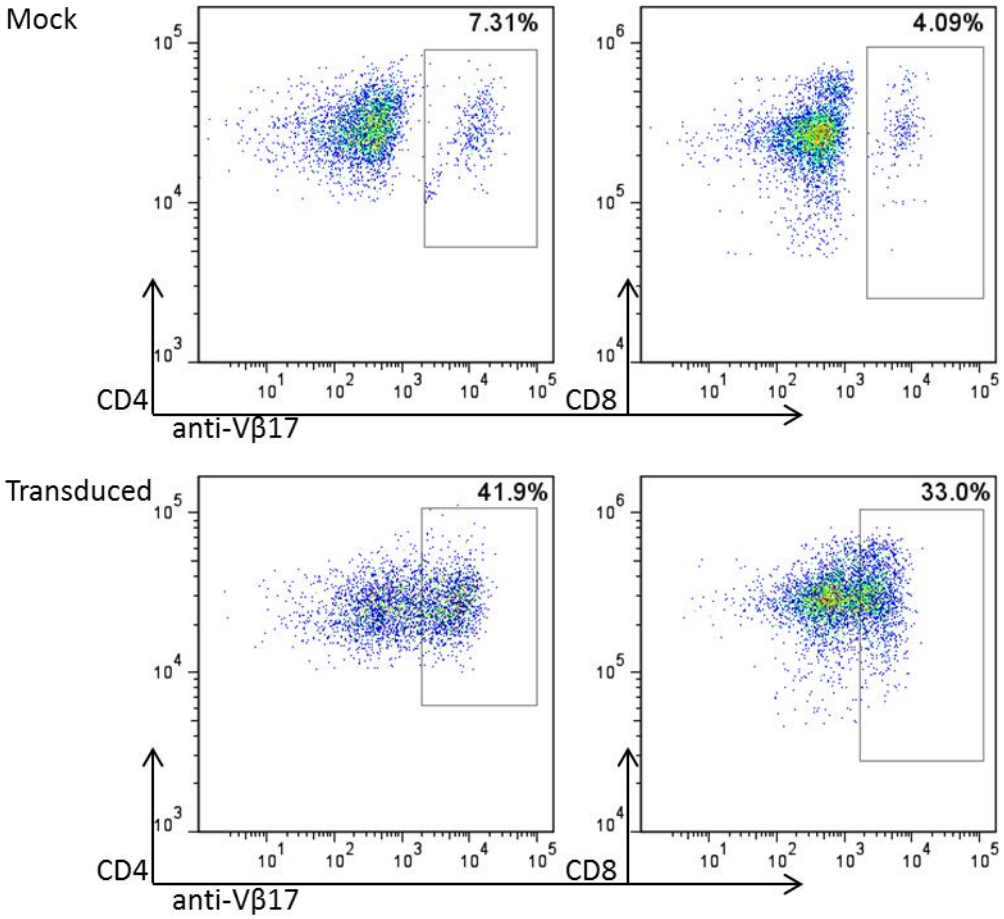
The TCR used here has been modified to include an additional disulphide bond within the constant domains to promote correct pairing between the exogenous alpha and beta chains. However, mispairing between the exogenous and endogenous TCR chains remains a safety concern [279]. As described in the introduction, this could potentially lead to the formation of TCRs with novel, undefined specificities, which have not been subject to central tolerance mechanisms in the thymus and therefore have the potential to target self-antigens [280] . The adoptive transfer of such undetermined specificities could theoretically cause unwanted toxic

and even lethal side effects. We therefore explored the level of TCR chain mispairing within our transduced T-cells.

To this end, we stained transduced and mock T-cell populations with the PRS-DR52b tetramer, and with the Vβ17 –specific antibody. Although the Vβ antibody will bind any TCR formed with this chain, the TCR must have both the correct alpha and beta chain in combination for the tetramer to bind. Our results showed that there are a small proportion of CD4+ and CD8+ T-cells in the natural T-cell repertoire which express Vβ17 and PRS-DR52b specific TCRs. The percentage of cells binding tetramer was almost identical to the frequency of Vβ17 positive cells, after mock Vβ17-positive cells has been subtracted (Figure 4.1), and this was true of every transduction in multiple donors. This strongly suggested that the exogenous Vβ17 chain was only pairing with the exogenous alpha chain to form functional TCRs expressed at the T-cell surface. Unfortunately, because antibodies to Vα5 are not available, it was not possible to determine whether the exogenous alpha chain was mispairing with endogenous beta chains.

Note that the tetramer staining also demonstrates that the transduced T-cells have the same specificity as the parent clone, and target the PRS peptide presented through HLA DR52b.

A)


Mock
7.31%
4.09%
CD4
anti-Vβ17
CD8
Transduced
41.9%
33.0%
CD4
anti-Vβ17
CD8

B)

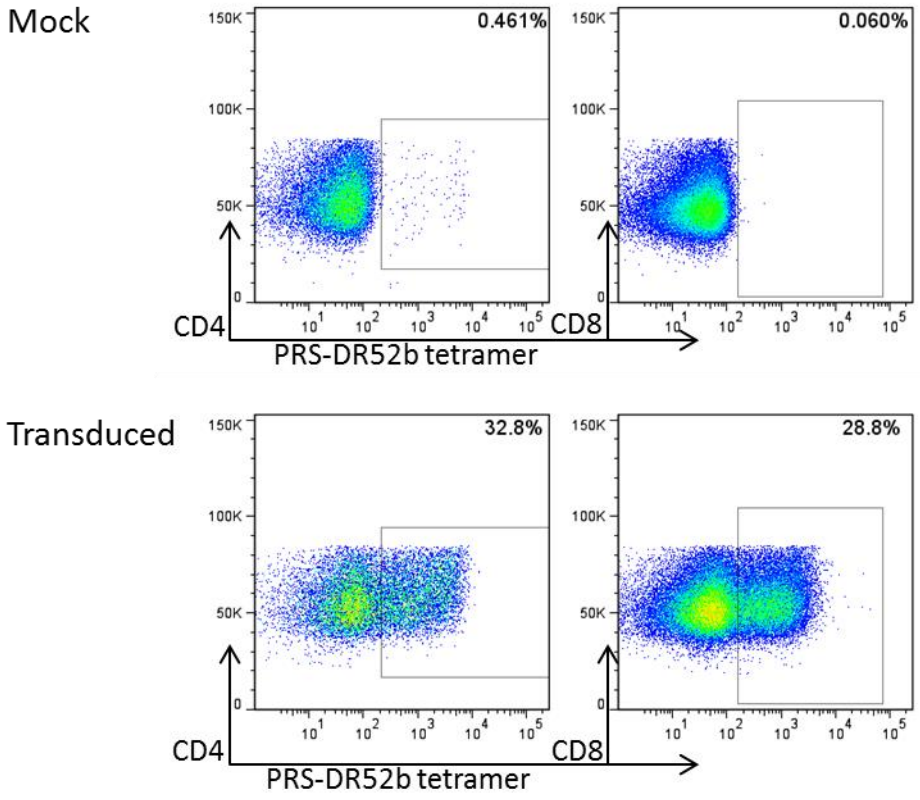


Figure 4.1 Surface expression of transduced TCR.

A) Transduced and mock T-cells were stained with anti-Vβ17 three days after transduction. B) Transduced and mock T-cells were stained with the PRS-DR52b tetramer three days after transduction. Cells were gated on live, CD3+ populations. Results are representative of four independent experiments. Percentage values shown represent the proportion of CD4 or CD8 T-cells that express either Vβ17 or have bound the PRS-DR52b tetramer.

The level of surface expression of the exogenous TCR on individual cells was more varied than that of the parent clone, as measured by the MFI of the tetramer staining of the transduced cells versus the parent c93 (Figure 4.2). This variance was likely reflective of

different transduction levels within different cells but could have also reflected different levels of competition for CD3 binding by the different endogenous TCRs [124]. T-cells with endogenous TCRs that bind CD3 weakly are more likely to express the exogenous TCR at the cell surface and vice versa [124]. Different endogenous TCR repertoires between donors may also explain the variation in staining intensity between donors.

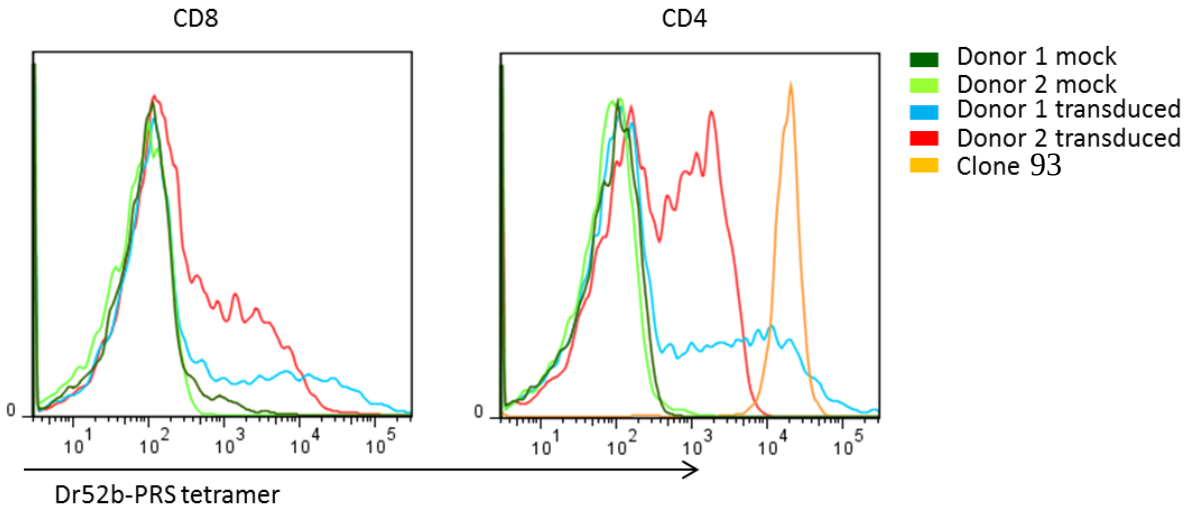


Figure 4.2 Tetramer staining of transduced cells and clone.

Cells gated on lymphocytes, live, CD4 or CD8. Two different transductions with different donor PBMCs shown.

4.2 The MHC CLASS II restricted TCR is functional in CD4+ and CD8+ T-cells.

4.2.1 Functional Avidity

The functional avidity of the transduced T-cells was defined as described in chapter 3 for the T-cell clones, as the concentration of peptide required to produce half a maximal response (Figure 4.3). Peptide titrations were performed by co-culturing $5x10^4$ total cells from PRS specific transduced T-cell populations with $1x10^5$ Ag876 LCLs, pre-loaded with titrating

peptide concentrations. The PRS epitope sequence is mutated in this strain and so there should be no background recognition of the target T-cell alone [260]. Clone 93 was diluted by mock-transduced autologous T-cells to ensure the same number of PRS specific T-cells were in both T-cell populations. Results showed that the transduced polyclonal T-cell line displayed a functional avidity of 4nM, which is very similar to though slightly lower than that of the parent clone (1nM) (Figure 4.3). The transduced population produced less total IFNγ than the clone, which could be due to some cells being exhausted after activation. Alternatively, it could be a consequence of the lower level of TCR on the T-cell surface when compared to the clone (see Figure 4.1). T-cells expressing low levels of surface TCR may not respond fully.

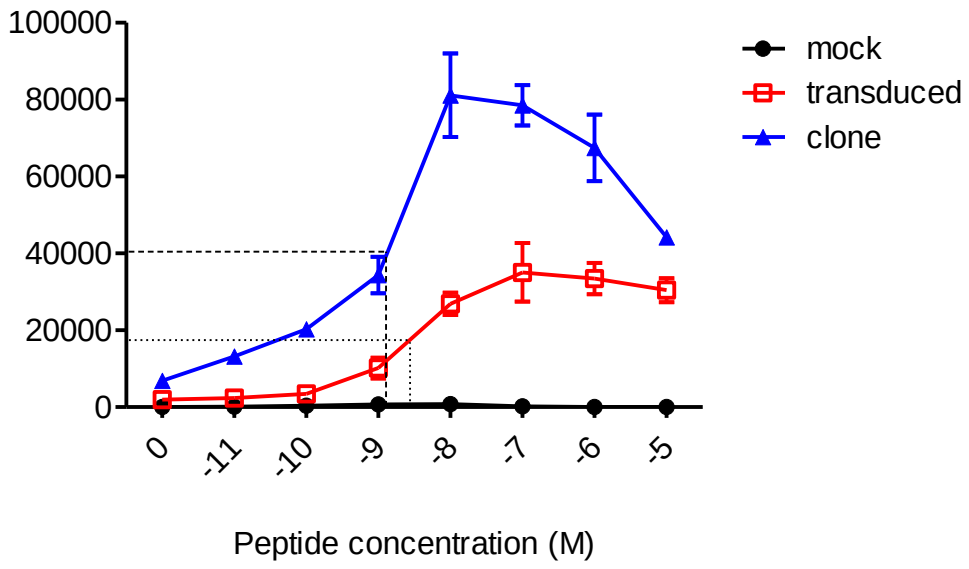


Figure 4.3 Functional avidities as determined by the concentration of peptide that produced half the maximal response.

Transduced cells were compared with the parent clone. Both transduced and clone cells have $5x10^4$ Vβ17+ T-cells per well. The transduced cells contained 25% CD8+ T-cells and 60% CD4+ T-cells. Results are representative of three experiments.

As the MHC class II restricted TCR was transduced into a total PBMC population, we separated the CD4+ and CD8+ T-cells to determine whether the TCR is functional in both subsets and if so, what their functional avidities against their target antigen are. Again, PRS was loaded onto B-cells infected with the type 2 EBV strain Ag876. After separating the two cell populations by negative selection with dynabeads, the resulting populations were stained with anti-CD4 and -CD8 antibodies to determine the efficiency of separation. As can be seen in Figure 4.4, separation was successful, with over 99% purity achieved here. In sorts that had over 1% contamination (containing cells which were depleted in the negative selections), controls were put in place (note, every sort had at least 98% purity). In those cases, mock cells were spiked with the equivalent frequency of contaminating transduced T-cells, to determine to what extant these contaminating populations produced Interferon gamma (IFNγ) in response to peptide loaded target T-cells. Any IFNγ produced from these control populations was subtracted from the results obtained with the sorted transduced T-cell subsets.

The results in Figure 4.5 showed that CD4+ T-cells had a slightly higher functional avidity compared to CD8+ T-cells, yet both populations were able to produce IFNγ in response to peptide. The difference in the functional avidities was likely due to the lack of CD4 co-receptor in CD8+ T-cells. As CD8+ T-cells have a functional avidity comparable to CD4+ T-cells, we suggest that the introduced TCR can function in CD4-negative T-cells, however introducing the CD4 co-receptor may be required to achieve optimal CD8+ T-cell avidity [237].

Levels of IFNγ produced in the mock cells were consistently lower than transduced populations. However, the small amount of IFNγ detected in some experiments scan be attributed to the naturally occurring EBV specific T-cells within the mock population, as

EBV positive donors were used as PBMC sources for transduction. As there was no IFNγ produced by mock or transduced cells alone, we have ruled out the possibility of background due to responses to fetal bovine serum and activation induced IFNγ production. Mock responses are shown here and then for clarity subtracted from every subsequent graph shown.

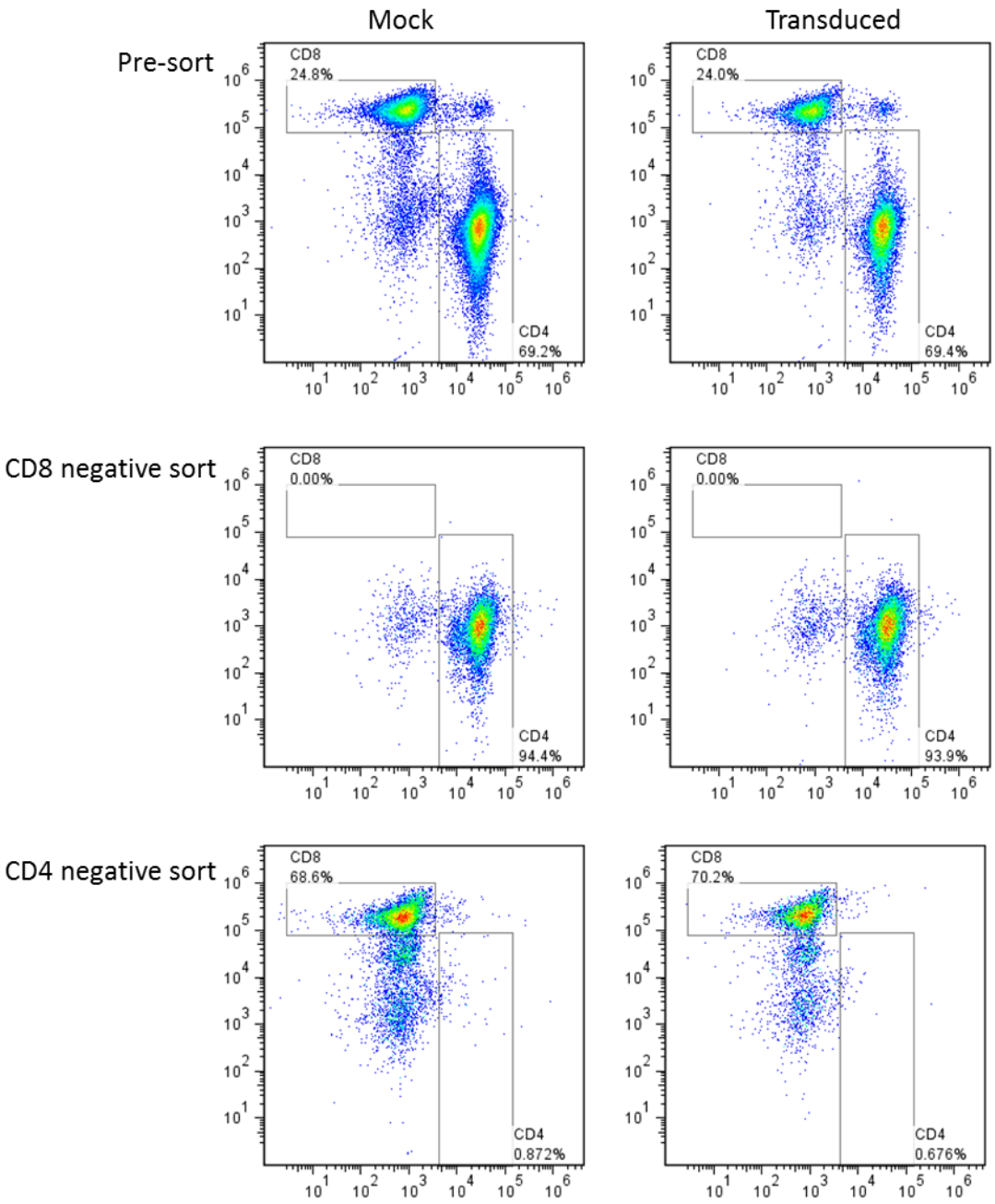


Figure 4.4 Negative sorting of CD4+ and CD8+ T-cells is efficient.

Graphs show pre and post sorted mock and transduced T-cells. Graphs are gated on lymphocytes, live, CD3, CD4 or CD8. Percentages show the percentages of CD4+ and CD8+ cells in pre and post sorted populations.

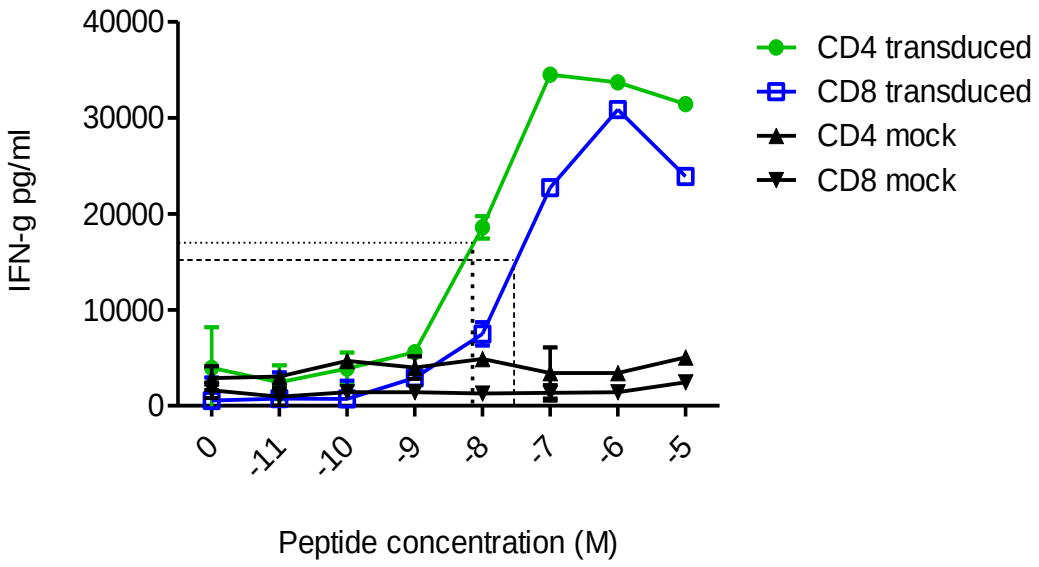


Figure 4.5 CD4+ and CD8+ T-cell functional avidities.

PBMCs were negatively sorted into CD4+ and CD8+ T-cell populations and tested. Vβ17+ percentage was normalised between the subsets, taking into consideration naturally occurring Vβ17+ cells seen in the mock population. Results are representative of three experiments.

4.2.2 Recognition of endogenously processed antigen and HLA restriction

To analyse the ability of transduced T-cells to recognise endogenously processed antigen, we performed an IFN-g ELISA with a panel of DR52b-matched and mismatched LCL targets, with each target pre-exposed to PRS-peptide or DMSO. By using a range of DR52b positive and negative LCL targets we were also able to confirm that PRS was being recognised exclusively in the context of DR52b in this panel of targets. As expected, transduced T-cells only produced IFNγ in response to DR52b positive LCLs, confirming that the response is restricted through this allele. The lack of response to a DR52b-matched LCL that carried the Ag876 EBV strain is consistent with this virus strain carrying a mutation in the PRS epitope sequence. Note however, that these cells, which are DR52b-positive, can be recognised when

coated with the PRS peptide sequence taken from the standard type I EBV strain B95.8. Crucially, the transduced T-cells produced IFNγ in response to DR52b+ LCLs even without pre-exposing the LCLs to the PRS peptide. This demonstrated that physiological levels of PRS naturally presented by EBV-infected B cells were able to sufficiently activate the T-cells, suggesting that the transduced cells should be functional as direct effectors against virus-infected cells *in vivo*.

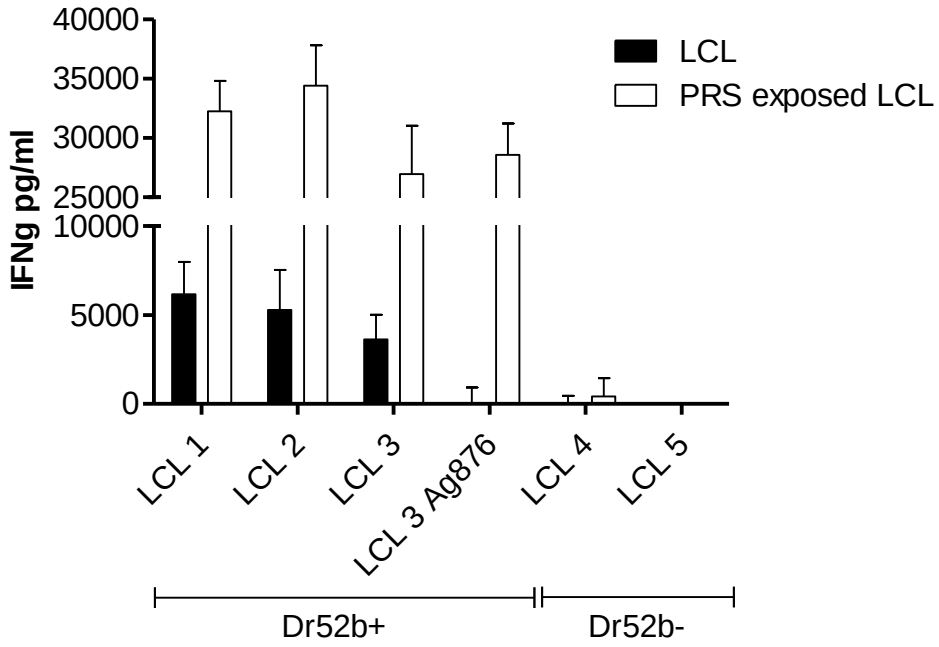


Figure 4.6 Recognition of EBV-transformed LCLs.

Transduction efficacy of the T-cells used in this assay was 18%. Transduced T-cells were co-cultured with DR52b positive or negative LCLs, pre-loaded with target PRS peptide or not. An IFNγ ELISA was performed on supernatant after 16 hour incubation. IFNγ produced by mock transduced cells and background IFNγ produced from T-cells alone has been subtracted. Results are representative of four experiments. All LCLs used here are infected with the EBV strain B95.8 unless otherwise stated.

4.2.3 Cytokine production in response to target T-cells

An increased frequency of CD4+ T-cells with capacity for multifunctional cytokine production is associated with improved control of some infections, including HIV and CMV [281-284]. We therefore sought to determine if transduced T-cells are multifunctional by ICS, as multifunctionality may also improve responses to tumours. T-cells were co-cultured overnight with peptide-exposed or DMSO-exposed LCLs, in the presence of BFA. The following day T-cells were analysed for the production of Interleukin 2 (IL2), IFNγ and tumour necrosis factor alpha (*TNFα*) by flow cytometry.

TNFα, as suggested by its name, is a cytokine with anti-tumour activity. It binds to TNF receptor (TNFR) 1 and 2 on target T-cells and induces downstream signalling pathways leading to apoptosis [285]. IL2 stimulates T-cell proliferation and induces activation of B and NK cells by signalling through its receptor, CD25 [286]. IFNγ up-regulates MHC class II on target T-cells, promotes cytokine production from DCs, activates macrophages and synergises with *TNFα* to induce tumour cell senescence [62, 287, 288]. These cytokines have all been used clinically for the treatment of different cancers, highlighting their anti-tumour effects [285, 289, 290]. Production of these cytokines by transduced T-cells would therefore suggest that the T-cells could have anti-tumour effects *in vivo*.

Intracellular staining allows us to look at multiple cytokines produced in any single cell. Logical gating strategies were applied to the flow cytometry results to identify cells that can produce one, two or three cytokines and these are described in materials and methods. Interestingly, both CD4+ and CD8+ transduced T-cells produced multiple cytokines in response to target T-cells. In Figure 4.7 we compared the frequency of cells producing one,

two or three cytokines, after the background from mock-transduced T-cells had been subtracted.

The results showed that, taking into consideration the transduction efficiency of 20%, a high percentage of transduced cells can produce *TNFα*, IFNγ and/or IL2 in response to autologous PRS-exposed LCLs (assuming that any cells producing cytokine were PRS-specific and responded in a TCR specific manner). Note that it was not possible to co-stain for the TCR alongside the cytokines, as T-cell activation leads to down-regulation of TCR on the cell surface. . These cytokines are commonly produced by Th1 T-cells, and so we suggest that at least some of our transduced T-cells display a Th1 phenotype. However, we have not tested for cytokines associated with other CD4+ T-cell subsets (e.g. IL17 secreted by Th17 cells), and as such cannot exclude that they may also be produced.

The majority of responding cells produced threat least two of these cytokines (Figure 4.7). Importantly, transduced T-cells also produced multiple cytokines when co-cultured with unmanipulated LCL. The percentage of cells producing one, two or three of these cytokines was greater in the CD4+ than CD8+ T-cells, which was likely attribuTable to the higher CD4+ T-cell functional avidity (Figure 4.5) and the inherent cytokine producing function of CD4+ T-cells. Indeed, increased cytokine production by CD4+ T-cells over antigen specific CD8+ counterparts has been observed not only by us but also by other groups [291]. CD4+ T-cells have also been shown to produce more cytokines that CD8+ T-cells when transduced with a MHC class I-restricted TCR [237].

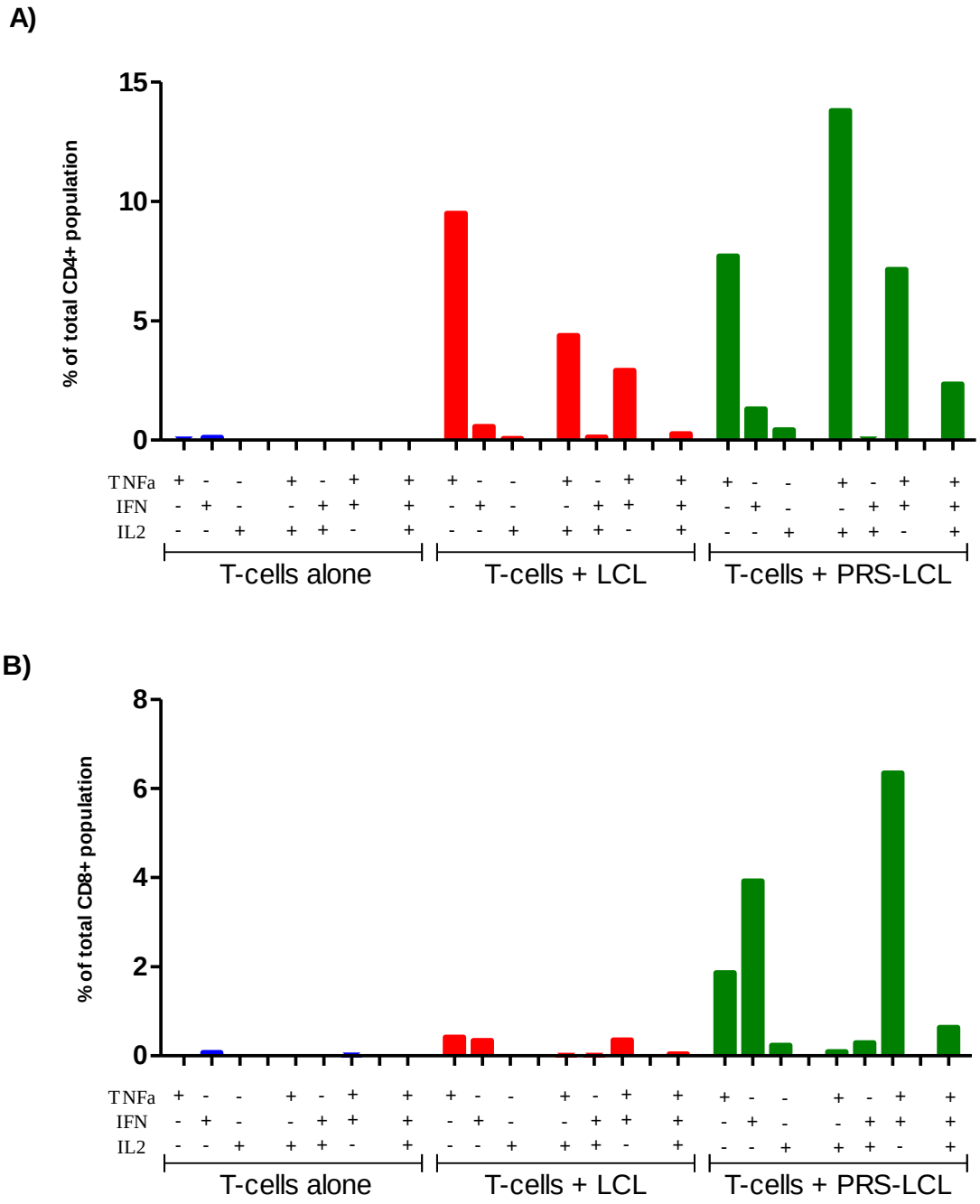


Figure 4.7. IFNγ, TNFα and IL2 production by transduced T-cells.

Transduced and mock transduced T-cells were co-incubated with LCLs, pre-exposed to epitope peptide or not, for 12 hour in the presence of BFAs. Cytokine production was measured by ICS following fixation and permeabilisation. Cells were gated on live, CD4+ or CD8+. Frequencies of mock transduced T-cells producing cytokines were subtracted .In the graph shown, the transduced CD4+ T-cells contain 20% more Vβ17+ cells than mock and the CD8+ T-cells are 23% Vβ17+ above mock. Results are representative of four experiments.

4.2.4 Proliferation Capacity

In order to efficiently transduce T-cells with retrovirus they must first be activated. Our protocol involves activating T-cells with 30ng/ml anti-CD3, 30ng/ml CD28 and 600U/ml IL2, for 48 hours. As activation will push cells to a more terminally differentiated phenotype, we have sought to confirm that the transduced cells still have proliferative capacity [292]. Indeed, repeated activations when expanding TILs prior to infusion has been linked to poor T-cell persistence which correlates to a shortened telomere length [293]. Proliferation is a key requirement for an effective adoptive transfer therapy, with clonal expansion of tumour specific T-cells increasing the number of T-cells that are able to respond to the tumour [294, 295].

To analyse the proliferative potential of the transduced T-cells we performed CFSE dilution assays (Figure 4.8). CFSE-labelled T-cells were co-cultured with irradiated (4000 rads) autologous LCLs, pre-exposed to epitope peptide or not and analysed after five days incubation. Results showed that activated transduced cells, as indicating by their Vβ17 expression, were able to proliferate in response to antigen *in vitro*, suggesting that they could proliferate *in vivo*. A proportion of Vβ17+ mock transduced cells also proliferated in response to LCLs, which we suggest is due to the presence of naturally occurring EBV specific T-cells within the population. Nonetheless, the proportion of proliferating cells is higher in transduced Vβ17+ cells. Almost all of the Vβ17+ T-cells in the transduced T-cell population proliferated in response to PRS-exposed LCL, and encouragingly, the proportion of these cells that proliferated to unmanipulated LCL was very similar.

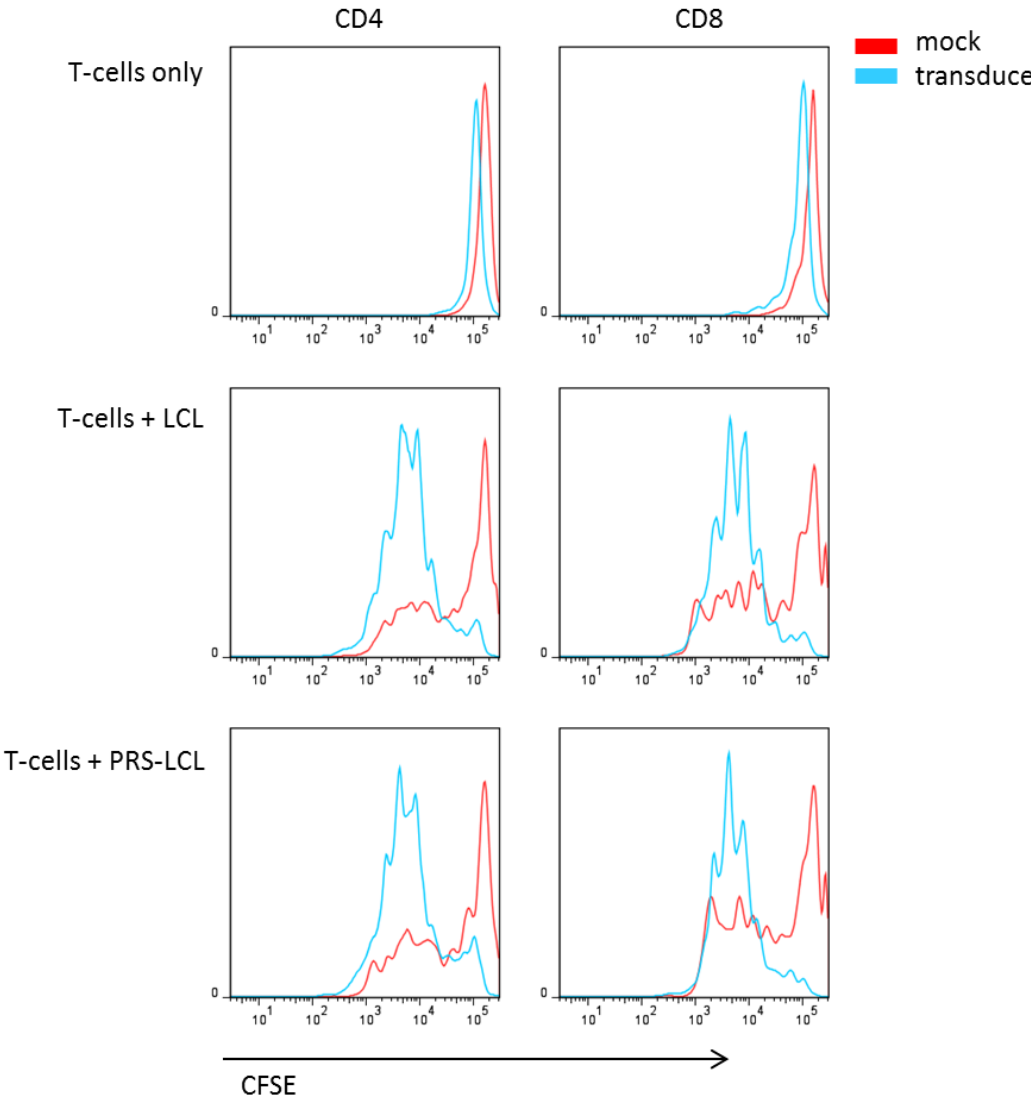


Figure 4.8. Transduced T-cells proliferate more than mock cells in response to antigen.

The graph shows T-cell proliferation in response to LCL and peptide epitope exposed LCL after 5 days co-culture. Results are representative of four experiments. Cells were gated on lymphocytes, live, cd4 or cd8, Vβ17. In the graph shown, the transduced CD4+ T-cells contain 33% more Vβ17+ cells than mock and the CD8+ T-cells are 32% Vβ17+ above mock.

4.2.5 Transduced CD4+ T-cells have the capacity to mature DCs

We have transduced T-cells with MHC class II restricted TCRs in order to harness the CD4+ response. Naturally occurring CD4+ T-cells are able to both orchestrate a broader immune response (including helping to promote and sustain CD8 T-cells) and mediate direct cytotoxic activity, making them very useful therapeutically. Therefore, our next experiments were designed to investigate whether the transduced T-cells have helper or effector functions, or both.

One way that CD4+ T-cells 'help' the immune response is by maturing dendritic cells (DCs). Through this maturation process, DCs are licenced to prime CD8+ T-cells, thereby ensuring CD8+ T-cells are fully activated and able to respond optimally to their target [296].

To assess the ability of the transduced CD4+ T-cells to mature DCs, CD8-depleted TCR transduced T-cell populations were co-cultured with immature DCs for two days. On day two, levels of surface CD86, CD40 and CD83 were measured on live, DC-SIGN+ cells (Figure 4.9). We observed that when iDCs were co-cultured with both mock and transduced T-cells, they upregulated CD83, CD86 and CD40, suggesting that the T-cells were capable of inducing a degree of maturation in the absence of peptide-MHC T-cell activation. This may have been due to T-cell activation prior to transduction, as activation up-regulates CD40L on T-cells [69]. When mock transduced T-cells were co-cultured with peptide exposed DCs a slight further increase in intensity of expression of the three maturation markers studied was always seen. This may be explained by naturally occurring PRS-specific T-cells within this mock-transduced T-cell population driving DC maturation in a peptide dependent manner. Importantly, transduced T-cells were much more efficient in maturing DCs in a peptide dependent manner than mock transduced T-cells. In fact, the intensity of expression of CD40

was higher than that of the positive control (namely DCs co-cultured with cytokines). CD83 and CD86 were also raised on PRS-pulsed DCs cultured with transduced T-cells, to similar intensities of expression as DCs co-cultured with cytokines. These results highlight the efficiency of transduced T-cells to mature DCs presenting their epitope peptide.

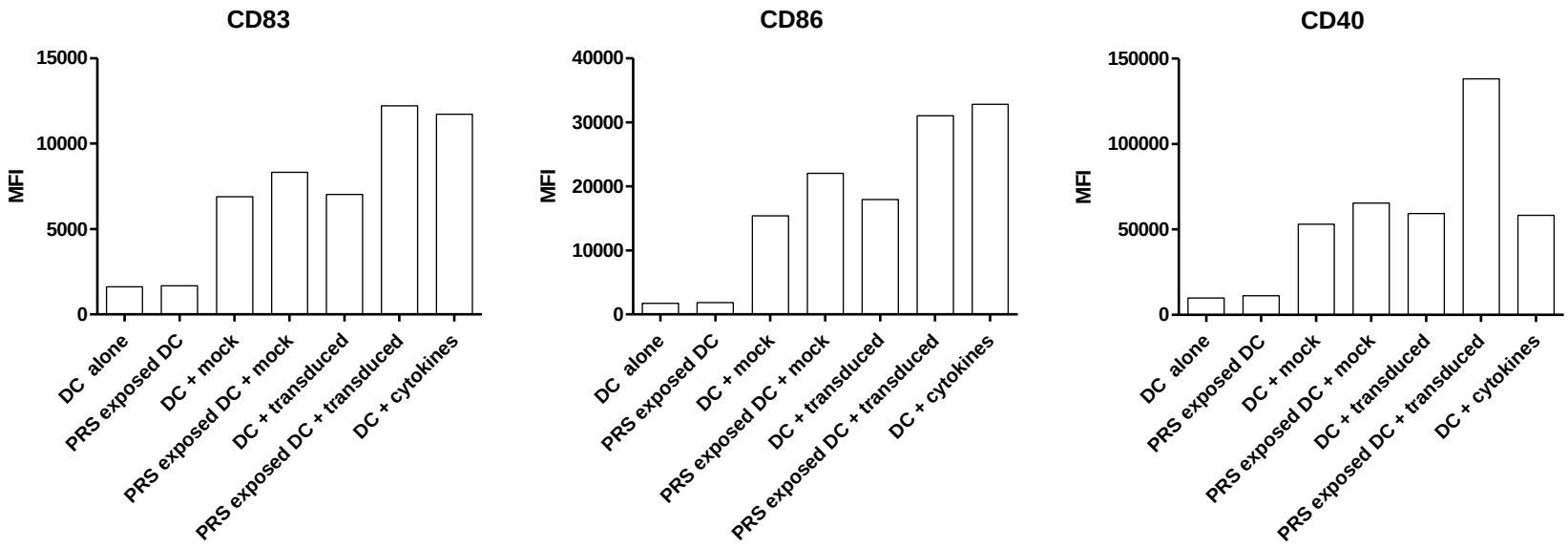


Figure 4.9. DC maturation is increased by TCR transduced CD4+ T-cells.

DCs were gated on live, DC-SIGN+. CD4+ T-cells were 28% Vβ17+ above mock in the graph shown. Results are representative of three experiments.

4.2.6 Cytotoxicity of transduced T-cells

After confirming that transduced CD4+ T-cells have retained their helper functions, we then went on to investigate the cytotoxic potential of transduced cells including both CD4+ and CD8+ subsets. CD4+ T-cells are able to induce tumour eradication by enhancing the immune response in a helper fashion (see introduction) but have also been shown to have direct effector functions including cytotoxicity. These direct effector functions can lead to eradication of tumours in immunocompromised mice, highlighting their therapeutic importance [76]. CD8+ T-cells are classical cytotoxic T-cells and are capable of eradicating tumours through this cytotoxic response [297].

The cytotoxic activity of transduced T-cells was determined by chromium release assays. Transduced T-cells were co-cultured with a range of DR52b+ and DR52b- LCLs, pre-loaded with ^{51} Cr, in the presence and absence of the PRS peptide (Figure 4.10). Results here show a 10:1 effector: target ratio after 5 hours co-culture. When DR52b+ LCLs were pre-loaded with PRS peptide, transduced T-cells induced high percentages of target T-cell lysis. This cytotoxicity was not seen when transduced T-cells were cultured with DR52b- LCLs, whether pre-exposed to peptide or not, confirming again that the response is DR52b restricted. Crucially, even in this short co-culture time of 5 hours, transduced T-cells were able to kill LCL target T-cells in the absence of peptide exposure. This indicated that the transduced T-cells could exert a direct effector response against LCLs presenting physiological levels of PRS antigen. As DR52b-positive Ag876 LCLs were not killed unless pre-exposed to PRS peptide, the response was confirmed to be PRS peptide specific.

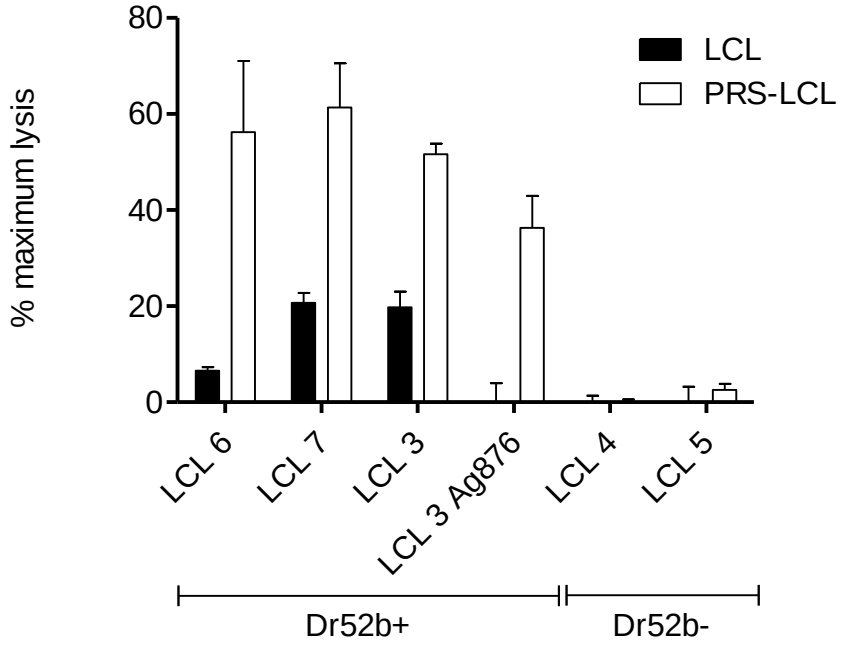


Figure 4.10. Killing of HLA matched or mismatched LCL targets, with or without peptide.

Graph shows a 10:1 effector: target ratio after 5 hour incubation. Results are representative of four experiments. In the graph shown, the transduced CD4+ T-cells contain 48% more Vβ17+ than mock and the CD8+ T-cells are 59% Vβ17+ above mock. LCL donors are labelled in accordance with those used in Figure 4.6. All LCLs used here are infected with the EBV strain B95.8 unless otherwise stated.

To establish which subset of T-cells were responsible for the cytotoxicity, in subsequent experiments we analysed the T-cells for expression of the de-granulation marker, CD107a, by flow cytometry. CD107a is a highly glycosylated membrane protein, that is used as a marker for de-granulation [298]. In resting cells, it is located within cytolytic vesicles, which contain perforin and granzyme [299]. When T-cells become activated, these cytolytic vesicles merge with the cell surface plasma membrane to release their cytotoxic granules and so CD107a that was originally located within vesicles now resides on the cell surface [300].

Results showed that after six hours CD107a was up-regulated in CD8+ transduced T-cells when co-cultured with autologous LCLs (either pre-exposed to PRS peptide or not) (Figure 4.11). A similar result was also seen in CD4+ Transduced T-cells, albeit at lower levels. This suggests that both CD4+ and CD8+ T-cell subsets mediate cytotoxic function in response to peptide-loaded LCLs but also in response to physiological levels of PRS peptide naturally expressed in an LCL.

We show that transduced CD8+ T-cells were more cytotoxic than transduced CD4+ T-cells. This provided further evidence that functional differences could be due to the different T-cell types and further rationale for transducing a bulk population of T-cells.

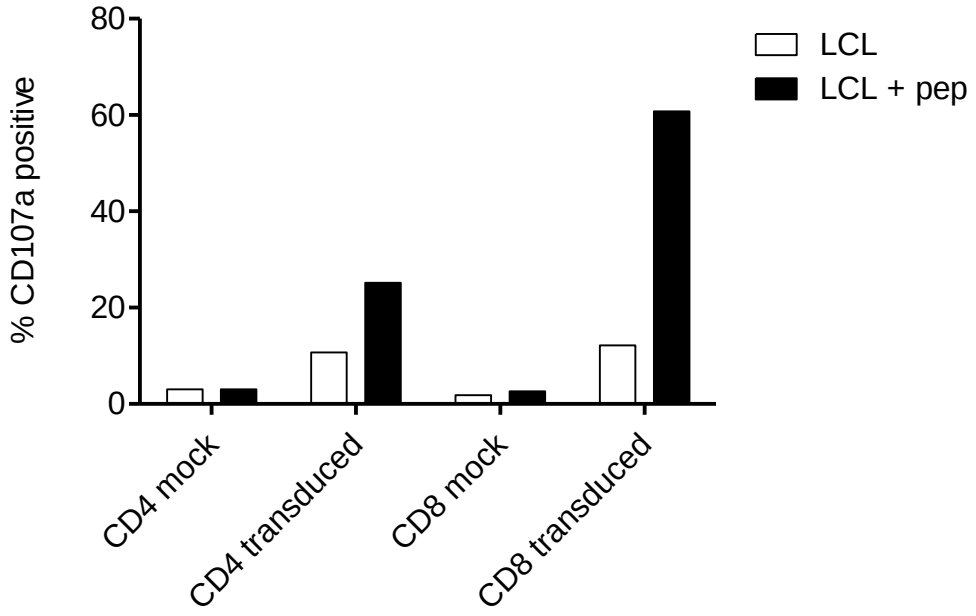


Figure 4.11. CD107a staining of T-cells after six hours of co-culture with autologous LCL and peptide loaded autologous LCL.

Cells are gated on live, CD3+. Results are representative of three independent experiments. In the graph shown, the transduced CD4+ T-cells contain 37% more Vβ17+ than mock and the CD8+ T-cells are 20% Vβ17+ above mock.

To confirm whether the cytotoxic activity of transduced cells seen in chromium release assays was sufficient to control the outgrowth of EBV infected cells, we set up outgrowth assays. These experiments span four weeks and so give us the opportunity to look at tumour control over a longer period of time *in vitro*. Within these experiments we used PBMCs from EBV seronegative donors as a source of T-cells for transduction, to avoid the complication of reactivation of naturally occurring EBV-specific T-cells specific for the immunodominant EBNA 3 family of viral proteins. In duplicate, we set up a serial dilution of LCLs, pre-exposed to PRS peptide or not, from 100,000 to 0 cells per well. To these we added either 100,000 TCR- or mock-transduced T-cells per well. After four weeks, the plates were

observed for wells that contained growing cultures. The results shown in Figure 4.12 indicated that the transduced T-cells are capable of controlling the outgrowth of an autologous LCL, even in the absence of exogenously added peptide. This suggests that the transduced T-cells could effectively control the outgrowth of tumours *in vivo*, which is critical to therapeutic success.

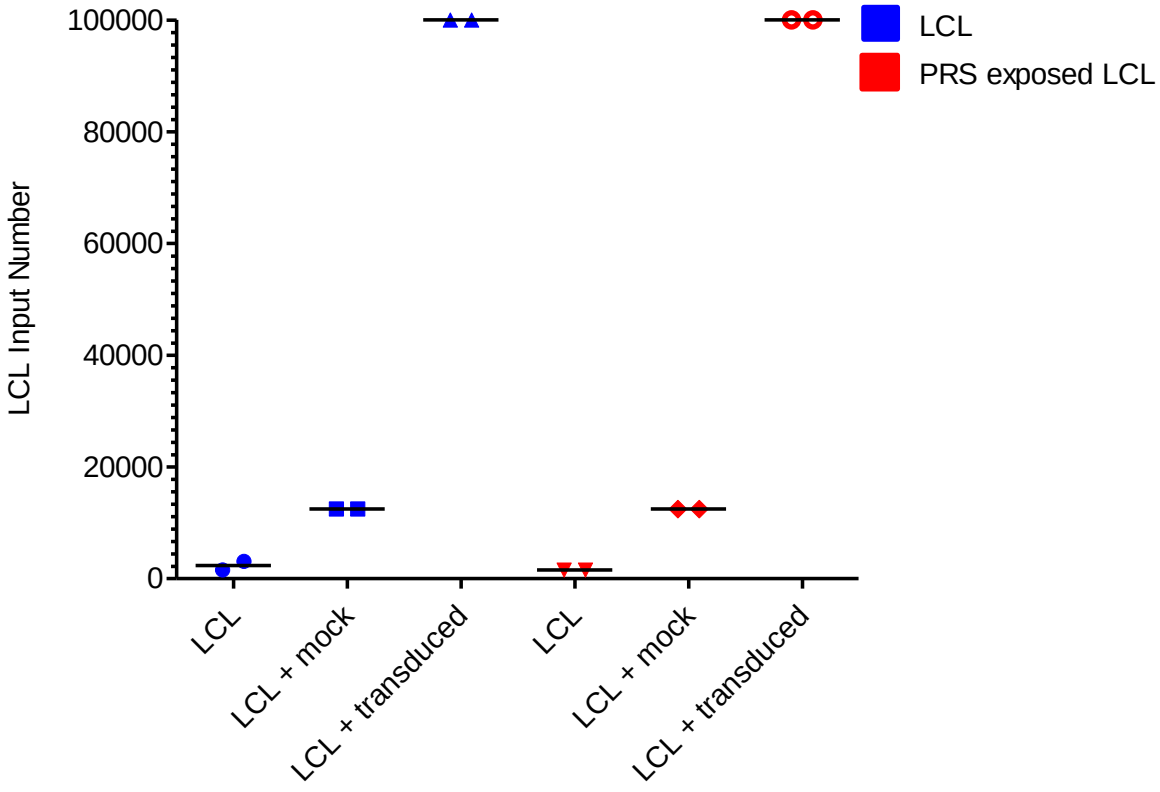


Figure 4.12. TCR transduced T-cells can control the outgrowth of LCLs in a long term assay both in the presence and absence of peptide.

In the graph shown, the transduced CD4+ T-cells contain 30% more Vβ17+ than mock and the CD8+ T-cells are 16% Vβ17+ above mock. Dots and squares represent the lowest input number of LCLs that resulted in growing colonies. Co-cultures were tested in duplicate and the black lines indicate the means. Results are representative of four independent experiments.

4.3 Discussion

To our knowledge, this is the first description of TCR gene transfer for a class II-restricted EBV-specific TCR. This could provide a novel therapy for the treatment of EBNA2 positive malignancies. In order to determine if this TCR gene transfer could be effective at clearing EBNA2 positive tumours, we have characterised the functional responses of transduced T-cells *in vitro*. We have transduced both CD4+ and CD8+ T-cells with this TCR and have investigated whether transduced T-cells act as helper and/or effector cells in response to target antigen.

4.3.1 Surface Expression of exogenous TCR

Our results show that the PRS-specific *MHC class II* -restricted TCR isolated during this work can be successfully expressed in both CD4+ and CD8+ T-cells to a similar level, and that at least the beta allele has paired correctly allowing functionality in both T-cell subsets. Here, we used constant chains which have been modified to include an additional cysteine residue on each TCR chain to allow for the formation of an additional disulphide bond. This has been shown to increase the exogenous TCR surface expression by increasing correct TCR pairing [301]. There are also other mechanisms available to reduce TCR mispairing, and each comes with both advantages and disadvantages. These include the use of murine constant domains, the removal of endogenous TCRs and generating single chain TCRs. However, to date, the optimal mechanism to reduce mispairing is still unknown, as to date there has not been a study directly comparing the different options.

4.3.2 TCR specificity

Through a range of functional studies we have demonstrated that both CD4+ and CD8+ T-cells transduced with our TCR can respond to target T-cells specifically, by p-MHC driven activation. This allows us to harness the functions of CD4+ *and* CD8+ T-cells against MHC class II restricted peptides.

Transduced T-cell specificity towards the EBNA2 derived PRS peptide presented in the context of HLA-DR52b has been validated in numerous assays. Tetramer staining of the transduced T-cells indicates that they are capable of binding to PRS when in complex with DR52b. These PRS specific T-cells are low or absent in the mock population, indicating the majority of tetramer-specific T-cells are carrying the exogenous, transduced TCRs. Additionally, the functionality of the transduced T-cells against DR52b-presented PRS was confirmed against LCL targets. Thus unmanipulated DR52b-positive B95.8 LCLs were recognised, with recognition increasing upon peptide loading, whereas DR52b-positive Ag876 LCLs were only recognised when pre-loaded with exogenous peptide.

4.3.3 TCR Cross Reactivity

As many TCRs are capable of binding to more than one peptide, there is the theoretical possibility this TCR might target other MHC:peptide complexes [302]. The TCR was derived from a T-cell from a healthy EBV positive donor, so because of self-tolerance mechanisms it should not respond to self peptides presented through HLA alleles carried by this donor. We cannot, however, rule out cross reactivity towards other epitopes not present in self tissues from this donor, or epitopes presented through HLA alleles that the donor does not carry. Kumari et al. have generated an HLA-A2 restricted TCR and tested it against a panel of cell lines expressing HLA-A2 without cognate antigen [303] and a similar approach could allow

us to investigate whether our TCR can cross react with other DR52b-restricted peptides. However, the approach is limited by the range of self-antigens expressed in the panel of cell lines. Whilst such cross-reactivity is a risk carried by all TCR gene therapies, it remains a theoretical risk in the context of naturally occurring TCRs. However, affinity maturation of TCRs alters the original TCR that was originally subjected to tolerance mechanisms, and has led to mortalities [144]. This off target toxicity highlights the benefit of using viral specific TCRs as they are naturally of high avidity and so do not need affinity maturation to function efficiently.

4.3.4 Avidities of transduced T-cells

Although the parent clone and transduced cells show very similar functional avidities, a factor that will alter the functional avidity between the parent clone and the transduced cells is the frequency of TCRs on the cell surfaces. Transduced cells express less exogenous TCR on the cell surface when compared to the parent clone (Figure 4.1). T-cells expressing less surface TCR may be less sensitive and therefore less responsive.

This reduced surface TCR expression could be a result of different gene expression levels in transduced cells. For instance, if the retrovirus integrates into a hypermethylated chromosomal area gene expression will be limited or shut off. Additionally, competition between endogenous and exogenous TCRs is also likely to be a factor that can lower T-cell avidity. To be expressed at the cell surface, the TCR must bind to the CD3 complex [304]. Therefore, the TCR with the highest CD3 affinity is more likely to be expressed at the cell surface.

To overcome this problem (which is theoretical here, due to the similar functional avidities between the parent clone and transduced T-cells), it is possible to disrupt endogenous TCR

gene expression prior to transduction, therefore limiting their surface expression. Provasi et al. have done this using zinc finger nucleases, which are directed towards specific DNA sequences and introduce double strand breaks. The process of non-homologous end joining is error prone and consequently, incorrect DNA sequences result, therefore disrupting the endogenous TCR sequences and resulting in lack of protein expression [305]. Using this method, T-cells that were pre-treated with zinc finger nucleases before transduction of TCR expressed more surface TCR and were superior in antigen recognition when compared to transduced T-cells without this treatment [125]. Alternatively, T-cells that have not yet developed endogenous TCRs can be transduced. Snauwaert et al. have successfully transduced postnatal thymus derived T-cells and adult hematopoietic progenitor cells for this purpose. These single TCR transduced T-cells effectively kill tumour cells *in vitro* [306]. Providing additional CD3 to transduced cells by co-transducing the CD3 complex could also overcome CD3 competition between endogenous and exogenous TCRs. This has been described to increase exogenous TCR expression up to ten fold [130].

Having taken these options into consideration, we decided to continue our studies with these T-cells, which have two different TCRs. We argue that the difference in avidity between the parent T-cell clone and transduced cells is relatively small, and the avidity of the transduced cells is still high in comparison to T-cell avidities that target self antigens, and is sufficient to mediate T-cell responses to EBV-transduced B cells *in vitro*. One of the main advantages of TCR gene therapy over other adoptive therapy techniques to target EBV positive lymphomas is its rapid T-cell preparation time. The methods described above to increase exogenous TCR surface expression all increase the time of T-cell preparation and the manufacturing cost. We therefore argue that these methods should only be considered if the benefits are substantial.

The lower avidity of transduced CD8+ T-cells when compared to CD4+ T-cells could be due to the lack of the CD4 co-receptor in CD8+ T-cells. Others have shown that when CD4+ T-cells are transduced with an MHC class I-restricted TCR the functional avidity can be lower than that of CD8+ T-cells transduced with the same construct. This can be rescued by co-transduction with a CD8 molecule, highlighting the requirement for the interaction of this molecule with MHC class I [307]. It is not, however, true in all cases, and it appears that some TCRs can function independently of their co-receptor, a phenomenon believed to be associated with high TCR affinity [308] [309]. The peptide titration assay performed here suggests that the transduced TCR has some CD4 dependency as transduced CD8+ T-cells have lower functional avidity than transduced CD4+ T-cells. To further investigate the co-receptor dependency of our TCR, CD4 interaction with class II could be blocked using a specific antibody and the effect of this could be tested in functional studies using the parent clone. Furthermore, CD4 could be co-transduced into the transduced CD8+ T-cells, to determine if this increases the functional avidity of the transduced CD8+ T-cells. Co-transduction of CD4 has been described by other groups and shown to improve cytotoxicity of CD8+ T-cells transduced with a class II restricted TCR [291].

4.3.5 Bystander activity

When looking at intracellular cytokine production in response to peptide loaded LCLs in CD4+ populations, there are more T-cells responding than there are transduced T-cells in the culture. This phenomenon could be attributed to bystander activity of T-cells [310]. As T-cells produce cytokines in response to target, they may activate other non-specific T-cells, as IFNγ, IL2 and *TNFα* are all immunostimulatory. These background responses may not be seen in non-transduced populations, as the majority of cells in culture there will not respond to LCLs, and it is conceivable that a threshold of cytokine production may be required to

activate non-specific T-cells. Such "bystander" effects also seen with CD107a staining, when CD8+ T-cells are co-cultured with LCL pre-exposed to peptide antigen.

In conclusion, transduced T-cells show helper effects *in vitro*. They can produce multiple cytokines in response to targets and mature DCs. Additionally transduced T-cells have shown effector responses *in vitro*. Transduced T-cells are capable of killing target T-cells as shown by chromium release assays, CD107a staining and long term outgrowth assays. All of these responses suggest that transduced T-cells have therapeutic potential.

CHAPTER 5

5 *In vivo* functionality of PRS-TCR transduced T-cells

5.1 Introduction

Work performed in chapters 3 and 4 has shown that PRS-TCR transduced T-cells can function effectively *in vitro*, with the ability to produce cytokines and kill target T-cells in response to physiological levels of antigen presented on virus-infected LCLs. These responses suggest that the transduced T-cells possess the functions required to control tumour growth, thereby indicating therapeutic potential. In this chapter, we have performed assays to determine the ability of transduced T-cells to eliminate EBV positive tumours *in vivo.*

5.1.1 Mouse model

For these experiments we used non-obese diabetic (NOD)/ severe combined immunodeficient (SCID)/ IL2 receptor gamma^{null} (NSG) mice. NSG mice have been developed over two decades, with SCID mice being bred first [311]. SCID mice have a loss of function mutation in the protein kinase, DNA activated, catalytic polypeptide (*Prkdc*) gene [312]. The Prkdc protein functions to resolve DNA strand breaks within V(D)J recombination of BCRs and TCRs [313]. Consequently, loss of function of this gene results in impaired lymphopoiesis and so SCID mice have very few B and T-cells. NOD mice were developed unintentionally through a breeding programme that aimed to generate cataract-prone subline mice. A mouse bred for this purpose was noted to have insulin dependent diabetes and further in-breeding from this strain resulted in mice which had high frequencies of insulin dependence [314]. It was later determined that NOD mice have polymorphisms at the insulin dependent diabetes loci 3. As the IL2 gene is located within this locus, IL2 expression is deregulated in NOD

mice [315, 316]. Consequently, this strain of mouse has impaired innate immunity, with reduced APC function, macrophage and NK cell activity and no complement system [317, 318]. SCID mice were crossed with NOD mice to generate NOD-SCID mice which had more severe impairments in adaptive and innate immunity than either strain alone [318]. However residual innate immunity is witnessed in this mouse model. To overcome this, NOD-SCID mice have been subjected to targeted mutations at the IL2-receptor gamma chain locus (*Il2rg*). This is a common gamma chain that is required for the generation of functional receptors for multiple cytokines, including IL2, IL4, IL7, IL9, IL15 and IL21 [319]. Mice lacking functional *Il2rg* have no NK cells and do not develop functional B- and T-cells [320]. Consequently, NSG mice are immunodeficient, lacking B- and T-cells. Additionally to this lack of adaptive immunity, NSGs have limited innate immunity, with no NK cells, reduced DC and macrophage functionality, and no complement systems [318].

This strong immunodeficiency renders NSGs suiTable mice to study PTLD. Prior to transplant, patients are treated with high doses of immunosuppressive drugs, chemotherapy and irradiation to ablate their immune systems [321, 322]. Consequently, PTLD patients are usually in the early stages of immune reconstitution, with little adaptive immunity [94].

By using NSG mice we are able to infuse human LCL and transduced T-cells without rejection. This is important because EBV does not establish infection in mouse B-cells. Additionally, NSG mice that are administered LCLs have been used widely as a model for PTLD and consequently this is an established model for testing therapeutic interventions for the disease [236, 323].

5.1.2 Pilot studies of optimal tumour dose

Firstly we set up a pilot experiment to determine the minimum amount of LCL required to generate consistent tumour growth and to produce palpable tumours by two weeks. This timescale was important because after the transfer of human T-cells there is a window of about four weeks to see an anti-tumour response, before the infused T-cells begin to cause GvHD, necessitating termination of the experiment [324]. Therefore we aimed to choose a dose of LCLs which would allow us to palpate tumours by two weeks or earlier, so that any anti-tumour responses could be determined by tumour size.

In the pilot experiment, we injected $2.5x10^6$ LCLs into three mice and $5x10^6$ LCL into three mice, subcutaneously (SC). We used the SC method of tumour administration as this was the stated method of administration on the project licence and we are able to measure tumour growth easily, by caliper. Importantly, all the LCLs used in this chapter are DR52b positive and had been stably transduced with luciferase before injection, to ensure that they could be detected by bioluminescent imaging of the mice after the injection of luciferin. Mice were monitored three times a week for tumour size (manually, by calliper) and once a week for tumour size by intravital bioluminescence imaging. Additionally, mice were monitored three times a week for signs of distress, including; weight loss, piloerection, disinterest in surroundings, lethargy, poor body condition, hunched back, swollen cheeks, sunken eyes and skin colour.

Mice were imaged by intravital bioluminescent imaging at day 7, 13 and 23, and then the experiment was ended due to tumour size limitations. No mice showed signs of distress.

As expected, both groups of mice developed tumours that were detecTable by intravital bioluminescent imaging, and the group of mice that received $5x10^6$ LCL had a larger mean

tumour size at each imaging point (Figure 5.1). However, there was intra-group variability in tumour sizes, indicating that more mice need to be used to show statistically significant results (Figure 5.2). Alternatively, more tumour cells need to be administered to overcome inaccuracies associated with injecting small numbers of cells or small volumes. In conclusion, injecting LCLs subcutaneously results in variable tumour growth and so we changed the mode of LCL administration for following experiments.

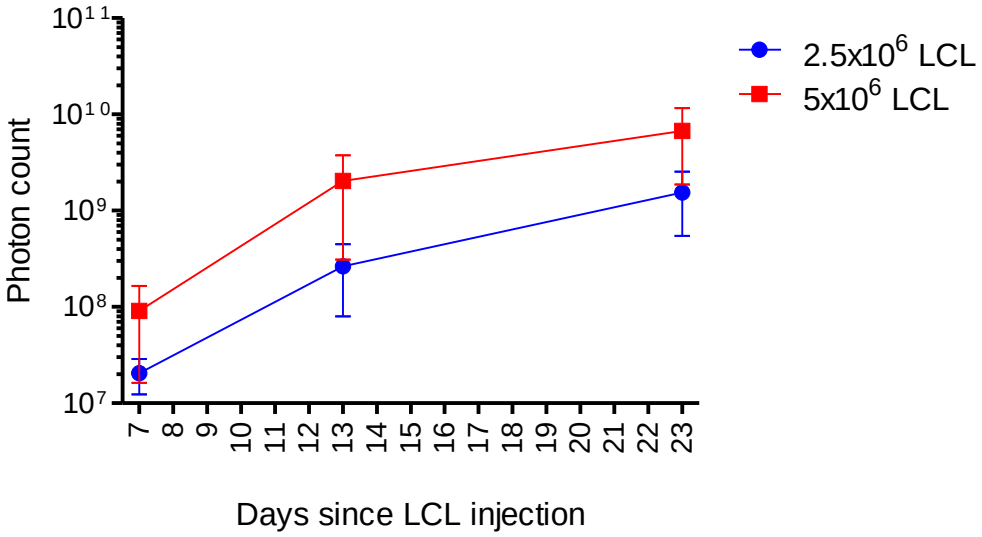


Figure 5.1. Tumour size as monitored by bioluminescent imaging.

Intravital imaging of subcutaneous tumours shows that mice developed tumours of different sizes, which grew with different growth kinetics. Graph is showing mean and SEM.

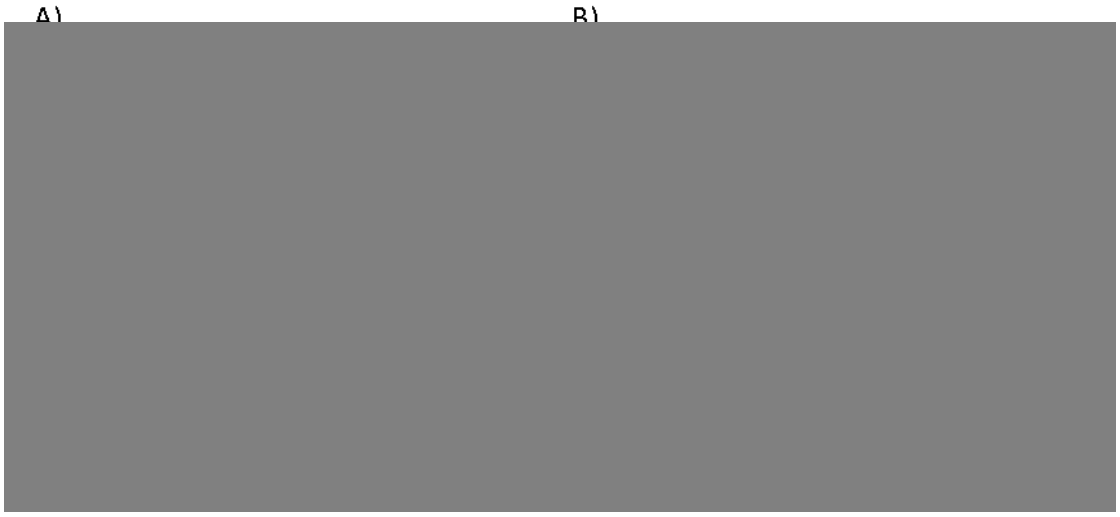


Figure 5.2. An image of mice injected with LCL subcutaneously.

In A) mice were injected with 2.5 million LCL. In B) mice were injected with 5 million LCL. The image was taken 23 days post LCL injection.

5.1.3 Testing intraperitoneal tumour administration

We next injected LCLs intraperitoneally (IP). Injecting LCL IP gives a much more physiological model of PTLD, as tumour cells can circulate the body, thus modelling the human disease [325].

Here, we injected 3 groups of mice with $0.01x10^6$, $0.1x10^6$ and $1x10^6$ LCL, IP, to determine the minimum amount of LCL that consistently resulted in tumour growth. Mice were monitored weekly for tumour growth by intravital bioluminescent imaging, and three times a week for the signs of distress previously mentioned.

Results show that no group had consistent tumour growth (Figure 5.3). At least $1x10^6$ LCL is required to visualise tumour development at time points before two weeks after tumour injection. At days 7, 11 and 14 tumours were undetecTable in mice which received $0.01x10^6$ LCL. Tumours were undetecTable at days 11 and 14 in mice which received $0.1x10^6$ LCL.

The detection limit can be set by determining background photon counts in mouse tissue that does not contain tumour. Mice which received $0.01x10^6$ or $0.1x10^6$ LCL were culled on day 21 as tumour was not consistently detected in these mice by this point and we were aiming to identify a dose of LCL which would produce visible tumours by day 14. At 28 days the experiment was ended, as we would not expect infused T-cells to be able to persist for longer than this time in mice without producing GvHD. No mice displayed any signs of distress during this time.

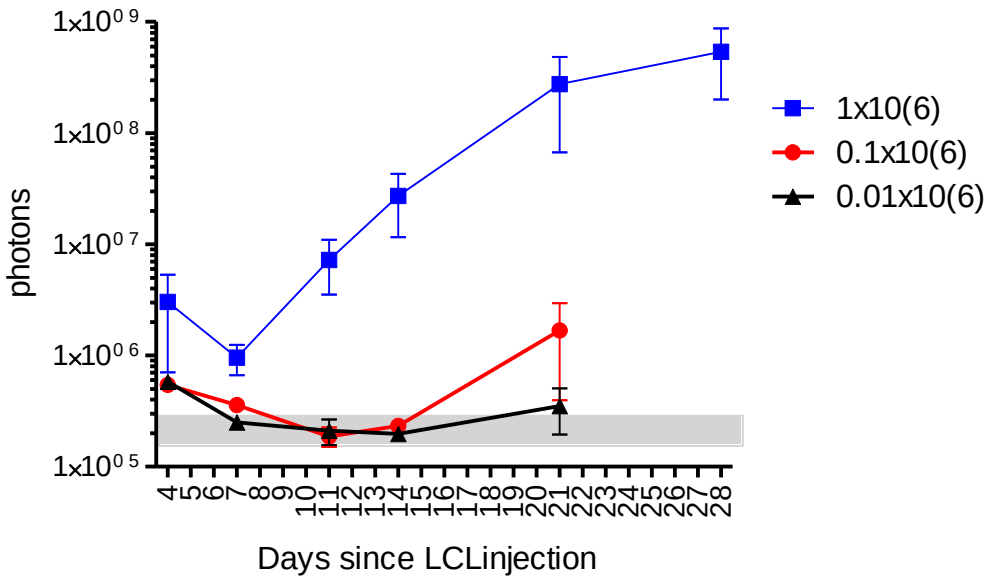


Figure 5.3. Tumour growth in mice as measured by Intravital bioluminescent imaging. Graph is showing mean and SEM.

The grey bar indicates the limits of detection.

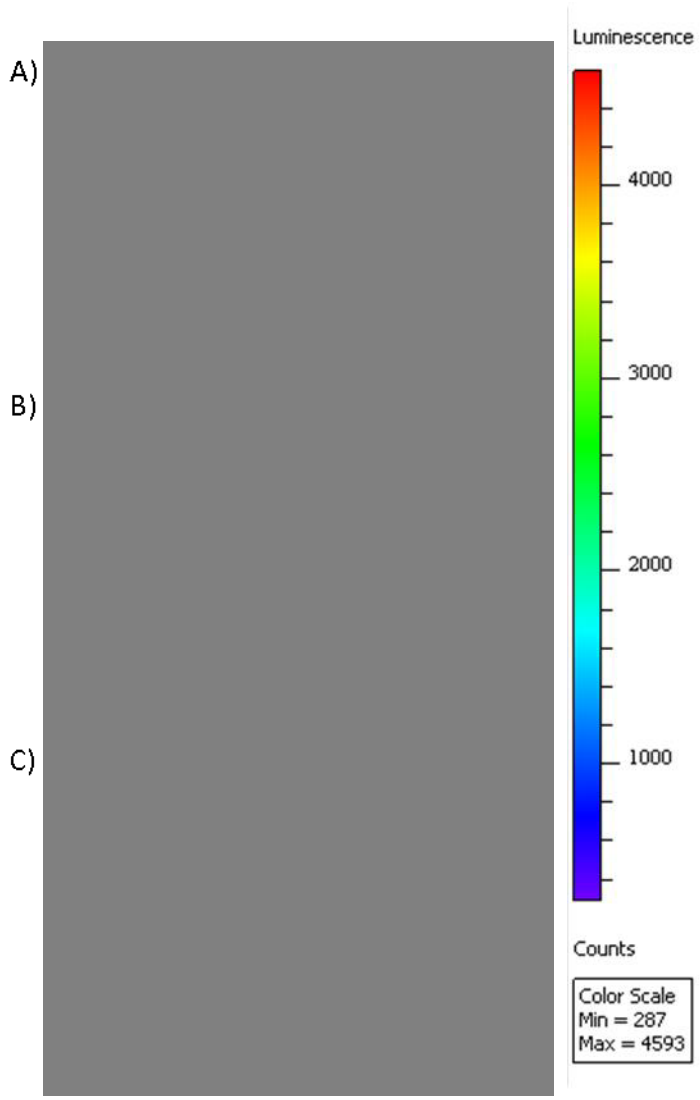


Figure 5.4. Tumour growth as measured byintravital bioluminescent imaging.

Here mice were injected with different numbers of LCL, inraperitoneally. Images were taken 21 days after LCL injection. A) shows mice which were injected with 0.01 million LCL. B) shows mice which were injected with 0.1 million LCL. C) shows mice which were injected with 1 million LCL.

5.2 Anti-tumour capacity of transduced T-cells

Next, we sought to determine the anti-tumour effect of transduced T-cells. The T-cells used throughout this chapter are from the same donor of the LCLs used, to avoid allogeneic responses against the LCL. Furthermore, the T-cells used are sourced from a healthy, EBV seronegative donor to avoid the possible complication of mock-transduced T-cells containing natural responses to EBV antigens (especially the immunodominant EBNA3 family of proteins).

5.2.1 Transduced T-cell function *in vivo*

We had previously shown that $1x10^6$ LCL was enough to form tumours in mice, therefore for the next experiment we decided to inject the mice with $2x10^6$ LCL, doubling the dose to hopefully improve consistency in tumour growth kinetics. Additionally, group sizes were increased to six mice per condition for more accurate results. All mice received LCLs, injected IP, on day 0. Six mice then received an IV injection of $1x10^7$ mock transduced T-cells, one hour after LCL injection, and six received $1x10^7$ transduced T-cells at the same time point. Of note, IV injections were performed by trained technicians, within the animal house. In this experiment, the transduced T-cells contained 27% more Vβ17 positive cells within the CD4+ subset, and 26% more Vβ17 positive cells within the CD8+ subset than mock transduced T-cells. As the transduced population consisted of 67% CD4+ T-cells and 18% CD8+ T-cells, the total number of transduced cells that were administered into each mouse was $2.3\ x10^6$.

Results showed a significant difference in tumour sizes in mice which received mock or transduced T-cells at day 7 ($p = 0.02$ using a two tailed Mann-Whitney U test which is used throughout this chapter). Mean tumour bioluminescence was 3.6 fold higher in mice which

received mock vs. transduced T-cells. However, this was not maintained to day 14 (p = 0.41) (Figure 5.5). All mice were culled at day 16 when they showed signs of distress. These included lethargy, sunken eyes and low body condition scores. The reasons for the distress symptoms are unknown. GvHD is unlikely the cause of distress as it does not normally develop until three to four weeks after T-cell injection [324]. Additionally, distress was unlikely to be caused by cytokine storms or tumour lysis syndrome. These occur when T-cells are so effective in killing target T-cells that the immune system is hyperactivated, causing heightened flu-like symptoms. As we did not see tumour control, this is unlikely the explanation. One possibility is that either the LCL or T-cells may have carried a low level of infection before infusion.

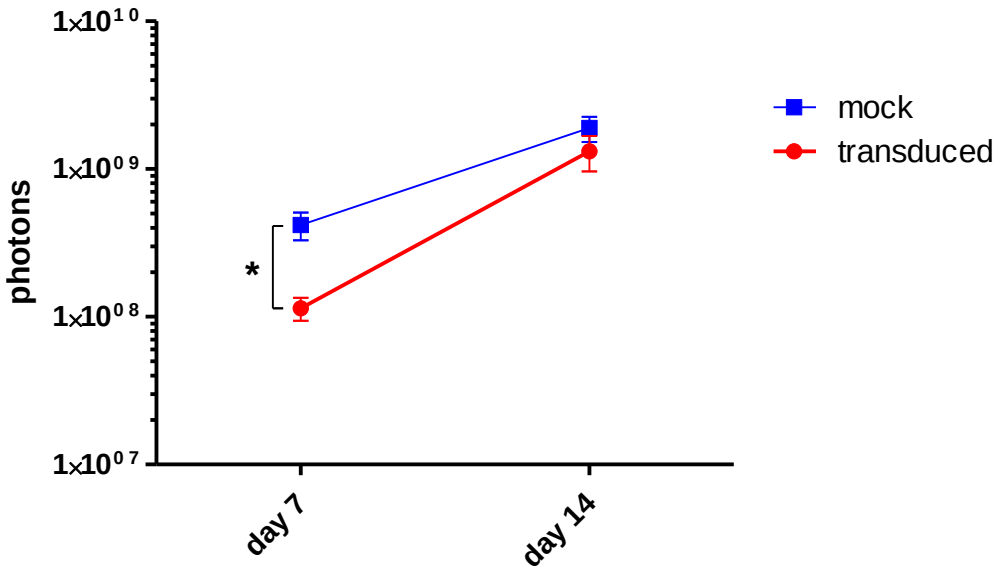


Figure 5.5. Intravital bioluminescent imaging

Imaging at day 7 and day 14 showed increase in tumour size over time, in mice which received both mock and transduced cells. Graph is showing mean and SEM.

Figure 5.6. Tumour growth after the administration of T-cells.

Intravital imaging of mouse tumours 7 days after LCL injection. In A) mice were injected with 2 million LCL and mock T-cells and in B) mice were injected with 2 million LCL and PRS-TCR transduced T-cells.

5.2.2 *Ex vivo* LCL phenotype

One possibility for the lack of continual tumour control is that surface MHC class II may have been selectively down regulated in LCLs when injected into the mice, as has been reported in another study using SCID mice [323]. To check if MHC class II is down regulated in the mouse models used here, we injected two NSG mice with $1x10^8$ LCLs (IP). On day 0 we gave one mouse an additional injection of 0.25mg/kg decitabine. This is a demethylating agent that is used in chemotherapy and was shown by Merlo et al. using a similar model to increase the expression of MHC class II on LCLs *in vivo* [323]. Injections were repeated twice on days 1, 2 and 3 in this mouse, to a total of 7 injections. The other

mouse received no drug. On day 4 both mice were culled and LCL was isolated from the peritoneum by peritoneal wash.

Results showed that surface MHC class II expression is partially down regulated on LCLs in NSG mice after 4 days. The median fluorescent intensity (MFI) of MHC class II staining on LCLs that have been *in vivo* for 4 days is two-fold lower than LCLs which have been cultured *in vitro*, however the MFI is still relatively high (Figure 5.7). Clearly with only one mouse per group these results are preliminary and would need repeating with larger numbers of mice. Additionally, it would be beneficial to set up assays with *ex vivo* LCLs and PRS-TCR transduced T-cells, to determine if the level of MHC class II down regulation seen reduces T-cell recognition, compared to recognition of the same LCL maintained *in vitro.* This lack of decitabine induced up-regulation of MHC class II clearly contrasts results published by Merlo et al [323]. It is plausible that decitabine can induce some level of MHC class II up-regulation, which is masked here by the low level of *in vivo* MHC class II down regulation.

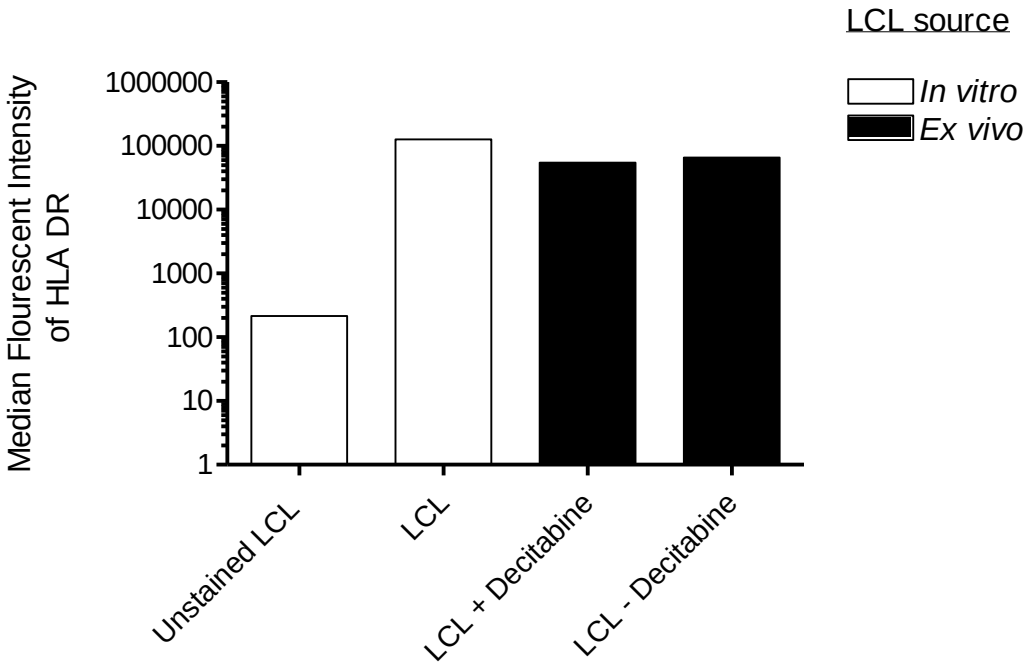


Figure 5.7 MHC class II expression on LCL that has been cultured *in vitro* or *in vivo*, with or without decitabine.

LCL was gated on lymphocytes, Live, MHC class I and HLA DR, after *in vitro* culture or isolation from mice post mortem.

5.3 Anti tumour effects of transduced T-cells *in vivo*

Due to time constraints a further experiment exploring the anti-tumour effect of PRS-TCR transduced T-cells was conducted in parallel with the previous decitabine experiment. Therefore it included the use of decitabine treatment in an attempt to restore MHC class II levels based on the published work of Merlo et al [323] (even though our preliminary data shown above suggests this was not necessary or effective). $2x10^6$ LCLs were injected IP into 14 NSG mice on day 0. All mice received a total of 7 injections of 0.25mg/kg decitabine, once on day 0 and twice on days 1, 2 and 3. On day 4, mice were intravitally imaged by bioluminescence imaging to determine tumour sizes. Mice were split into three groups; 2

control mice which would not receive T-cells, and two groups of six mice which would receive either PRS-TCR or mock transduced T-cells. The mice receiving T-cells were monitored at day 4 for tumour growth and split into two groups with similar tumour sizes. $15x10^6$ transduced or mock T-cells were then injected into each mouse IV. Of the infused T-cells, 50% of the population were CD4+ and 29% of the population were CD8+. Both CD4+ and CD8+ transduced T-cells had 15% Vβ17 positive T-cells more than their non transduced counterparts. Therefore the group of mice that received the PRS-transduced T-cells received a total of $2.25x10^6$ genetically engineered T-cells. Mice were monitored three times a week for signs of distress, as previously mentioned, and once or twice a week for tumour size by intravital bioluminescence imaging. Additionally, tail bleeds were performed once a week to investigate the persistence and/or expansion of adoptively transferred T-cells.

5.3.1 T-cell persistence and expansion *in vivo*

Results from the tail bleeds showed that adoptively transferred T-cells persisted in the peripheral blood of mice throughout the experiment (Figure 5.8). Between days 8 and 19 post LCL injection, both total CD4+ and CD8+ T-cells gradually proliferate in mice which received mock or transduced T-cells. CD4+ T-cell and CD8+ T-cell proliferation is exponential after this time point, which is a classical characteristic of GvHD. In particular, GvHD is characterised by a proliferation of CD8+ T-cells, which is observed here [326].

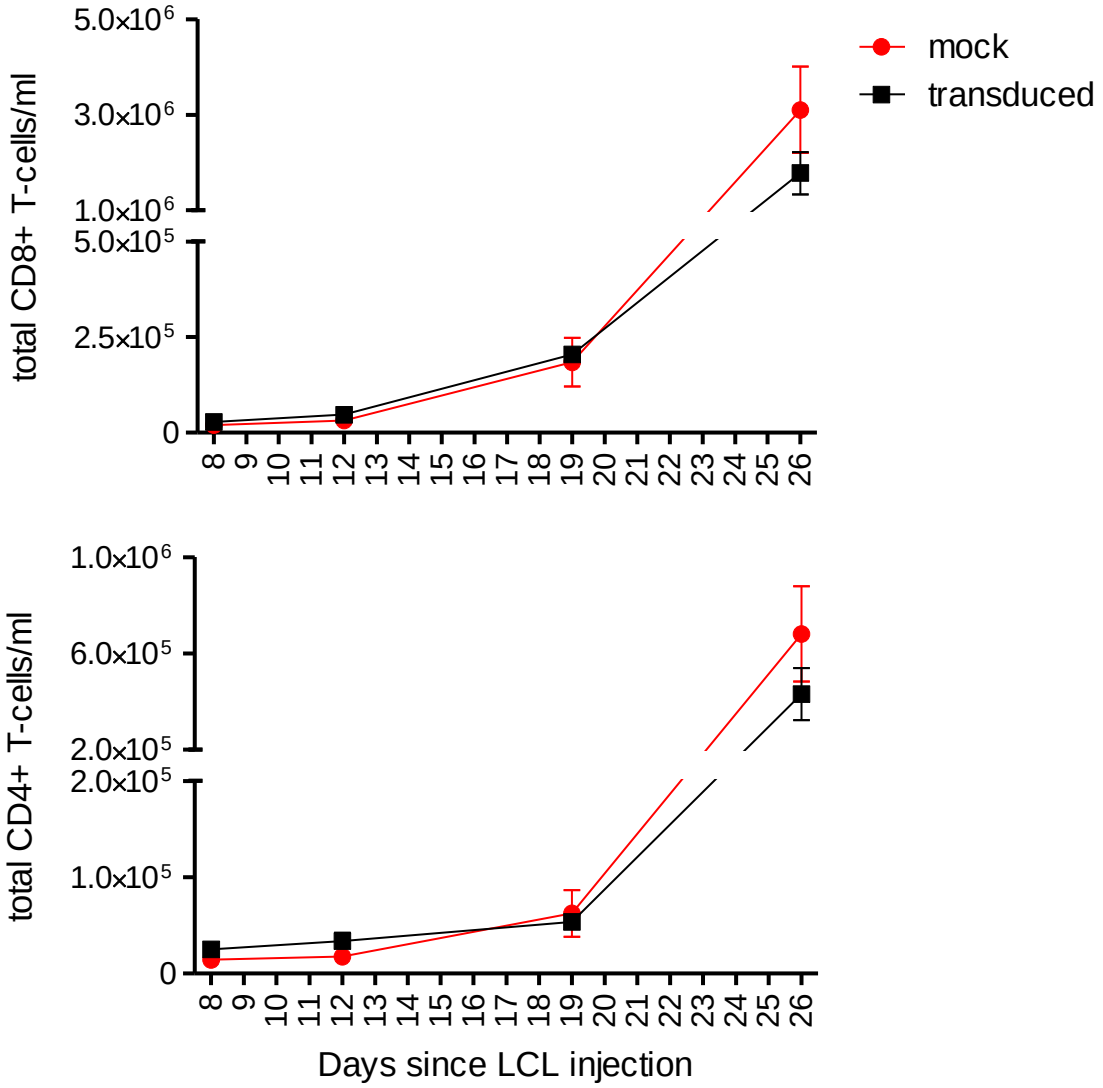


Figure 5.8 Total numbers of CD4+ and CD8 T-cells in mice.

T-cells were gated on single cells, lymphocytes, live/dead, CD4+ or CD8+ T-cells. The number of cells per ml of blood was calculated by the administration of a known number of Flow Cytometer Cell Counting Beads to each sample prior to analysis on the BD LSR II flow cytometer. Graph is showing mean and SEM.

The T-cells from the peripheral blood were further analysed for surface expression of Vβ17, to identify whether transduced cells were selectively expanding, which would be an indication of anti-tumour responses.

The percentage of Vβ17 positive cells was consistently higher in mice which received transduced T-cells, when compared to mice which received mock T-cells (Figure 5.9 b and d). This is expected, as the transduced T-cells contained more Vβ17 positive cells than the mock when analysed prior to infusion. The percentage of Vβ17 positive T-cells in mice which received transduced T-cells increases slightly between the time of T-cell injection and day 8, at which point the percentage drops until day 19. After this time point, the percentage of Vβ17 positive T-cells begins to increase slightly in both CD4+ and CD8+ T-cells. The loss of Vβ17 positive T-cells between day 8 and 19 in transduced CD4+ and CD8+ populations may reflect a greater tendency for successful transduction of more highly differentiated cells which are also more likely to succumb to senescence [327]. Alternatively, transduced cells could be selectively lost in peripheral blood because they are leaving the hematopoietic system to attack tumour cells in tissues. To determine if transduced T-cells were leaving the hematopoietic system, a reporter system such as the luciferase system used here could be used to track the T-cells. Had tumours been visible, solid masses, tumours could have been isolated and sections could have then been stained for the presence of human T-cells *ex vivo*.

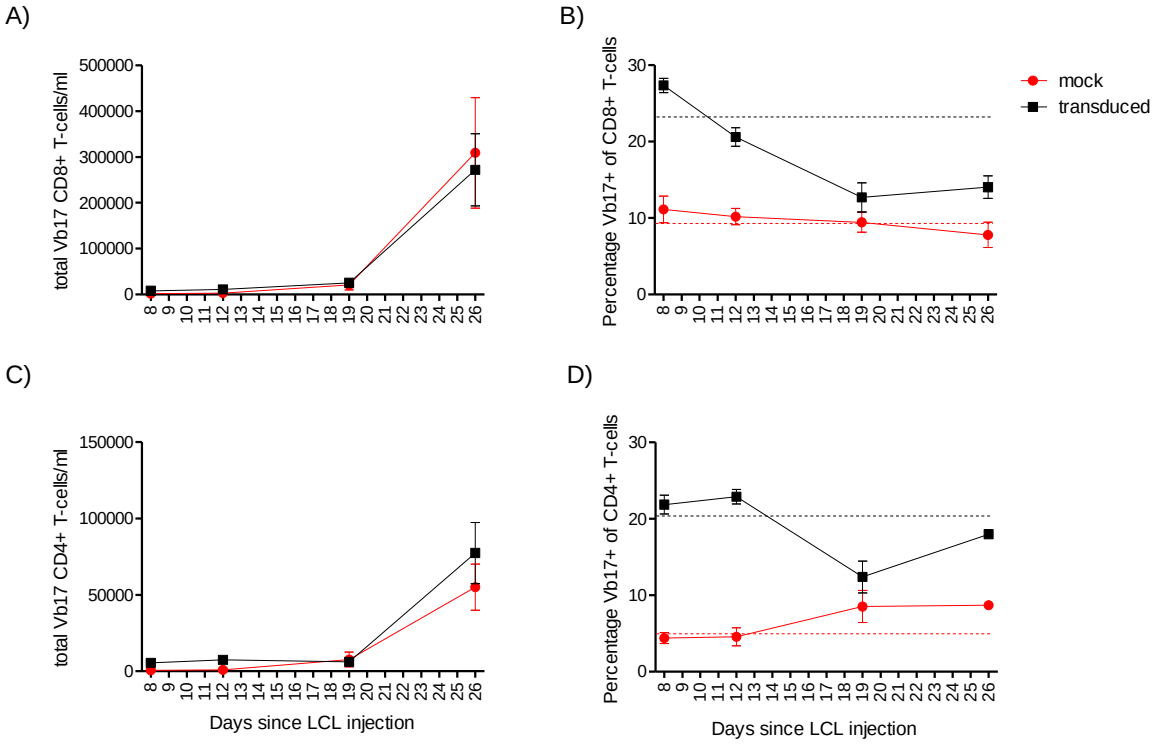


Figure 5.9. The total number and percentage of Vb17 positive T-cells per ml of peripheral blood. Graph is showing mean and SEM.

Figure 5.9 a) shows the total number of Vb17 positive CD8+ T-cells in mice which received mock or transduced T-cells. B) shows the percentage of total CD8+ T-cells which are Vb17 positive in mock and transduced populations. The dashed line shows the percentage of Vb17 positive T-cells *in vitro*, prior to injection. C) and D) show the same results but for CD4+ T-cells.

5.3.2 Tumour sizes

Intravital imaging results showed that in this experiment, there was no statistical difference in the ability of the transduced T-cells to control tumour growth above the mock T-cells (p value of 0.17 when comparing tumour sizes between the groups at day 28 using a two tailed Mann-Whitney U test). Importantly, both groups of mice appear to show some level of

tumour control after day 11 (Figure 5.10). Mean tumour sizes appeared similar in both groups of mice between day 11 and day 18 and then tumours appear to shrink in both groups, but at a faster rate in mice which received PRS TCR T-cells. Interestingly, this correlates with the time point at which the percentage of Vβ17 positive T-cells begins to increase in the blood (Figure 5.9), and so could possibly have been the beginning of tumour control by transduced T-cells. This indication of anti-tumour response is not statistically significant in either group of mice, as the difference between tumour sizes at day 11 and 28 in mice which received mock and transduced T-cells, produced p values of 0.57 and 0.06, respectively. It is clear, however, that whilst significance was not quite reached, mice which received transduced T-cells showed more tumour control than mice which received mock T-cells. This suggests a trend of tumour control by transduced but not mock T-cells.

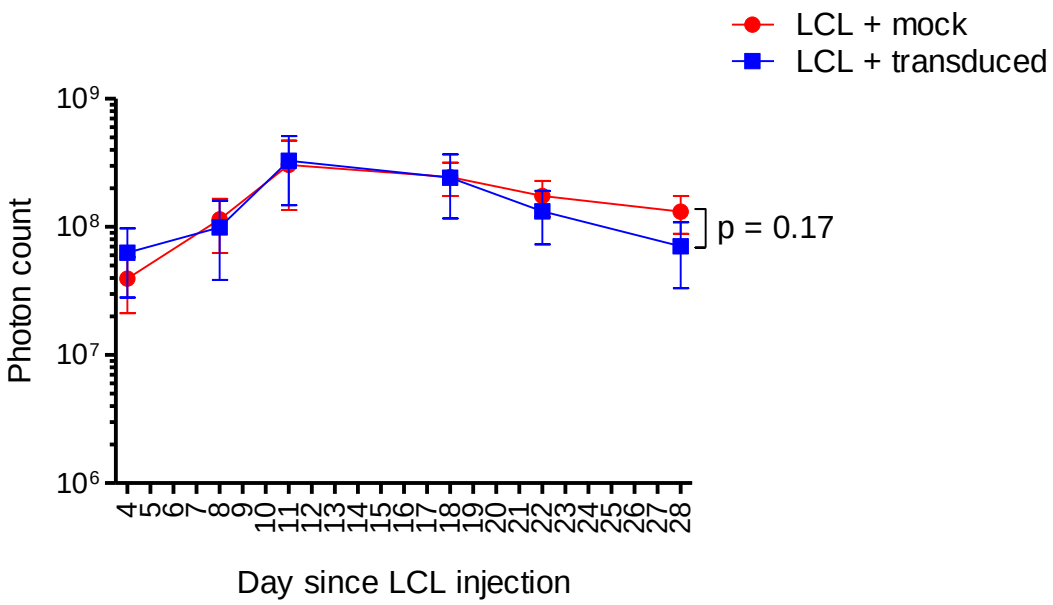


Figure 5.10 Tumour sizes as measured by intravital imaging.

Intravital bioluminescent imaging of mice which received LCL and mock or transduced T-cells, at different time point since LCL injection. Graph is showing mean and SEM.

Figure 5.11 Intravital imaging of mouse tumours.

Mice were injected with 2 million LCL and multiple injections of decitabine followed by mock or transduced T-cells at day 4. This Intravital image shows tumour sizes 28 days after LCL injection.

Figure 5.11 shows intravital imaging of mice at day 28 and clearly shows that one mouse in the group which received transduced T-cells is an anomaly, with much larger tumour than all other mice in this group. Interestingly, when this mouse was removed from analysis, the difference in tumour control between the groups of mice is even more pronounced (Figure 5.12). Statistical significance is very nearly reached between the groups of mice by the end of this experiment ($p = 0.055$). Further experiments could use larger groups of mice to determine if there is a statistical difference in tumour control.

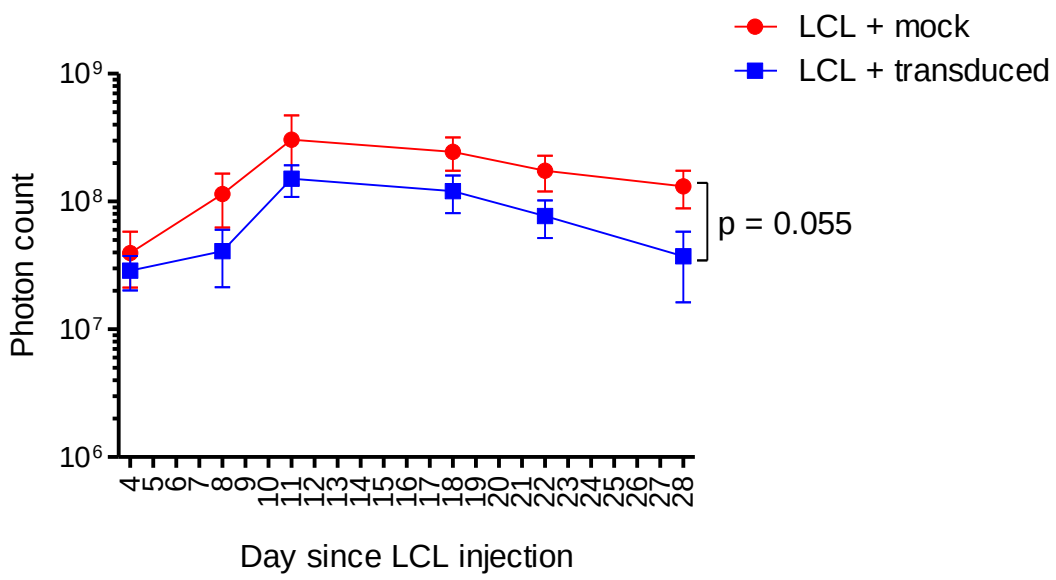


Figure 5.12 Tumour sizes as measured by intravital imaging with anomaly removed

Intravital bioluminescent imaging of mice which received LCL and mock or transduced T-cells, at different time point since LCL injection. Here, the mouse with the largest tumour has been removed from the group of mice which received transduced T-cells. Graph is plotting the mean and SEM.

We ended the experiment at day 28 since the mice were showing signs of distress including piloerection, lethargy, disinterest in surroundings, swollen cheeks and face washing (likely due to GVHD). Therefore, unfortunately, we could not monitor the tumour sizes after day 28 to determine if transduced T-cells are capable of controlling tumour over a longer time period.

5.3.3 *Ex vivo* LCL phenotype

After termination of the experiment, LCLs were recovered from the peritoneum post-mortem to investigate their phenotype after *in vivo* growth in the presence of T-cells. We were interested to test the hypothesis that transduced, PRS-specific T-cells could have selectively eliminated LCLs which retained sufficient surface levels of MHC class II but LCLs that might have lost class II expression would still have grown in these mice. *Ex vivo* staining showed that LCLs from mice which received mock or transduced T-cells had similar levels of surface MHC class II (Figure 5.12) indicating that there was no selective killing of *MHC CLASS II*+ LCL by the engineered T-cells.

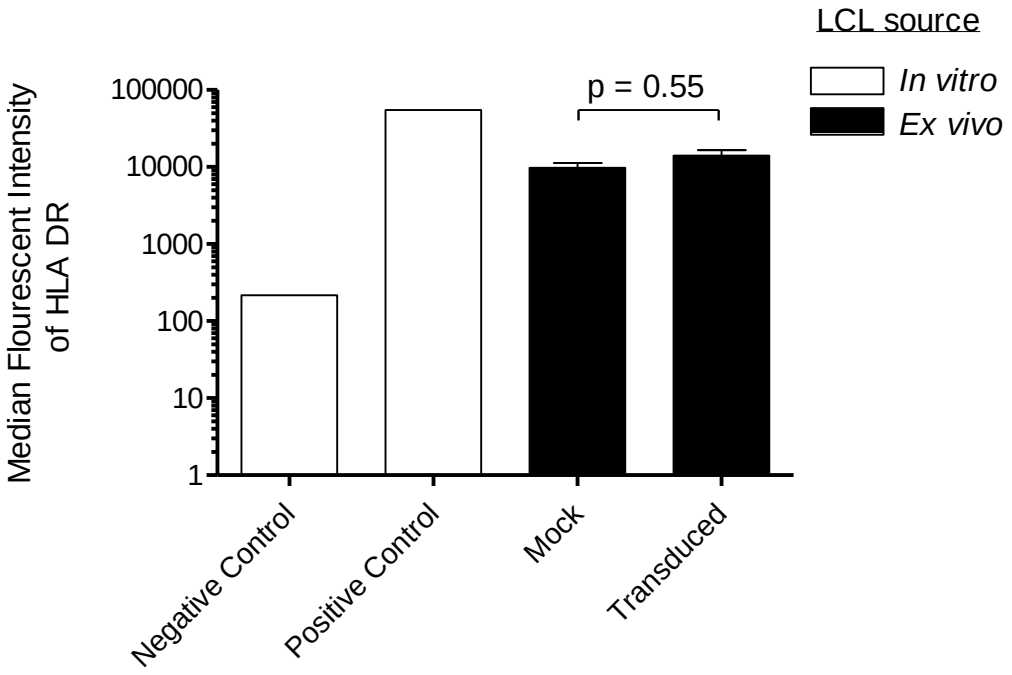


Figure 5.13 HLA DR expression on LCLs restored from mice post-mortem.

LCL was gated on lymphocytes, Live, MHC class I and HLA DR, after *ex vivo* growth in the presence of mock or transduced T-cells. The negative control was unstained LCL which was cultured *in vitro* and the positive control was stained LCL which was cultured *in vitro.*

5.4 Discussion

This chapter focused on assessing the potential *in vivo* anti-tumour effects of T-cells transduced with the PRS-TCR, using NSG mice injected with EBV-transformed LCLs as a model for PTLD. This is important because control of tumour outgrowth in such pre-clinical models can indicate the therapeutic potential of transduced T-cells. The earlier *in vitro* assays had shown the therapeutic potential of PRS-TCR transduced T-cells, through their cytokine production and cytotoxicity against target T-cells. Additionally, transduced CD4+ T-cells maintained a helper function, as shown by their ability to mature DCs. We therefore hypothesised that transduced T-cells with such functions would show some control over tumour growth *in vivo.*

5.4.1 Tumour control by transduced T-cells

Results obtained here give an indication that T-cells genetically engineered to express the PRS specific TCR may be able to control tumour growth *in vivo* better than activated, unmodified T-cells. Work performed by others has shown that naturally occurring EBV specific T-cells can control LCL growth *in vivo,* in immunocompromised mice. However in these experiments EBV specific T-cells were administered the same day as LCL injection [236, 325]. Consequently these experiments were testing the ability of T-cells to control tumour growth, rather than to eliminate established tumours. Here we have begun preliminary work to investigate the ability of EBV specific T-cells to control tumours 4 days after they were injected.

Although there was not a clearly significant effect on tumour growth, both experiments which included T-cell administration showed a trend towards better tumour control using transduced rather than mock T-cells. It is possible that transduced T-cells did not have a long lasting

anti-tumour effect because the tumour microenvironment suppressed immune responses. Some EBV positive PTLDs have been shown to be PD-L1 positive by IHC (19/26 cases) [328]. It is therefore not inconceivable that PD-L1 was expressed here and suppressed T-cell responses during the experiment. Post Mortem analysis of LCLs for PD-L1 expression could be investigated in future experiments.

Figure 5.10 shows that the tumour size increased in mice which received mock or transduced T-cells until day 11, at which point tumours begin to shrink in both groups of mice. Two mice in this experiment received LCL and decitabine but no T-cells , and tumours in these mice did not shrink. In addition, previous assays (injecting differing numbers of LCLs) have never shown spontaneous tumour reduction. This suggests that the tumour could be being controlled to different extents by both mock and transduced T-cells, rather than shrinking spontaneously.

The possible low level of control of tumour by mock T-cells in the final experiment was unexpected, especially considering that mock responses to LCL in short term *in vitro* assays, with T-cells from the donor used here, were consistently low. There are multiple possible explanations for this which could be further investigated. Firstly, it is possible that the T-cell donor has seroconverted recently and now carries EBV. The last EBV serology testing for this donor was performed a year before these experiments and so this could be possible. If so, mock T-cells which are reactive towards EBV antigens could have expanded over time *in vivo* and controlled tumour growth. Note that if this was the case, transduced T-cells should also contain these naturally occurring EBV specific T-cells with additional PRS specific engineered T-cells, and so should produce greater tumour control.

Alternatively, mock T-cells could have been responding non-specifically, due to activation with anti CD3 and anti CD28. Mock T-cells could also have been responding to cellular

antigens, upregulated in B-cells after transformation. LCLs have previously been shown to be targeted by CD4+ T-cell clones which do not recognise EBV derived antigens [329]. Again, if this was occurring I would expect to see the same non-specific activation or cellular antigen derived responses from transduced T-cells. As transduced T-cells additionally have PRS specificity, anti-tumour responses should be greater in this T-cell population.

Importantly, the difference in tumour sizes in mice which received mock and transduced T-cells increased from day 11 onwards. The last intravital image at day 28 showed that tumours were smaller in mice which received transduced T-cells, as can be seen clearly in Figure 5.11. Additionally, when one anomaly was removed, statistically significance was very nearly reached ($p = 0.055$ when tumour sizes were compared at day 28 between mice which received mock and transduced cells). This trend for greater tumour control by transduced T-cells that increases with time implies that had the experiment ran for longer, statistically significant differences in tumour sizes would have been observed.

5.4.2 Limitations of model

There are certain limitations that are recognised with mouse models, and these need to be taken into consideration when interpreting results. Firstly, there are inaccuracies with injection of cells. IV injection of material is technically challenging; If any of the sample is not injected into the vein the mice will receive a reduced dose of T-cells, which could affect the results [330]. Similarly, IP injections need to be accurate, to prevent injected cells entering the intestine or the bladder [331]. If this happens injected cells would be excreted, leaving mice with different T-cell numbers. For the work performed here highly experienced technicians were employed to inject T-cells IV and I injected LCLs IP only after sufficient training and practise.

Intravital bioluminescent imaging is a useful tool in the analysis of cells in mice. As cells transduced with luciferase emit light after the introduction of luciferin, signals can be quantified. This is less subjective than measuring tumour sizes by caliper. However, the depth of the tumour must be taken into consideration. Mouse tissues quench bioluminescent emission signals from luciferase transduced cells and so the same signal can appear stronger if the cells are closer to the skin and weaker if the cells are more internal [332]. However, signal increases or reductions can be compared accurately in the same mouse at different time points.

5.4.3 Future work

As time constraints only allowed for preliminary *in vivo* work, there are many future experiments that could be performed here to gain a better understanding of the function of PRS-TCR transduced T-cells *in vivo*.

Firstly, it would be beneficial to perform a dose escalation study to determine if anti-tumour effects could be seen if more genetically engineered T-cells were administered. In the final experiment performed here which looked at the anti-tumour response of PRS-TCR transduced T-cells, a total of $2.25x10^6$ transduced T-cells were infused. Many clinical trials aim to infuse between 10^{10}-10^{11} T-cells, which equates to approximately $2x10^7$ for a 20g mouse. Therefore we could perform a dose escalation study, increasing the number of infused genetically engineered T-cells nearly tenfold. However because the transduction efficacy in this experiment was 15%, to do this here we would have needed to infuse many more than $2x10^7$ T-cells per mouse to reach this number of PRS-TCR transduced T-cells. This would make GvHD more likely and could results in GvHD occurring quicker.

To overcome this, we would need to use cells that are more efficiently transduced. Previously we have had transduction efficiencies of 35%, which would be beneficial here. Alternatively, transduced T-cells could be sorted to obtain a pure population, using an anti-Vβ17 antibody followed by magnetic separation or fluorescence activated cell sorting (FACS).

In this experiment we were also limited by the amount of blood that we had available as a source of T-cells; we were only able to take up to 200ml of blood from a healthy donor. To obtain more T-cells, buffy coats could be used, which often contain more than a billion T-cells. However the disadvantage of this is that many products would need to be collected and tested to find one which is DR52b positive and EBV negative, and then LCLs from that cone would need to be generated and transduced with luciferase. This would be a time consuming process, and was not possible here due to time constraints.

Additional future work to evaluate the longer-term anti-tumour effect of PRS-TCR transduced T-cells against LCL targets could use a mouse model that is not limited by GvHD, such as humanised mice. New-born NSG mice can be injected with human HSCs from DR52b positive donors, which develop in the mice to produce humanised immune systems. T-cells could be isolated from spleens of humanised mice such as these to use as a transduction source. This would prevent GvHD, as the T-cells have been tolerised to mouse proteins during development [333]. Figure 5.10 shows that transduced T-cells have superior anti-tumour responses over mock T-cells. By using a humanised mouse model the experiment could be extended to test the hypothesis that this anti-tumour effect would reach significance with extended time.

It would be interesting to track the infused T-cells *in vivo* using, for example, T-cells transduced with a luciferase construct. Luciferase is derived from multiple sources such as fireflies, sea pansies or bacteria. Different sources emit different bioluminescent lights and so

could be used in combination within one mouse to track both T-cells and tumour cells [334]. It would be interesting to see if the infused T-cells contact the tumour.

Finally, the differentiation status of transduced T-cells could be investigated. It is possible that transduced T-cells are more efficiently activated during the transduction procedure than non-transduced T-cells and therefore could enter a replicative senescent state quickly *in vivo,* limiting tumour control. Indeed, more differentiated effector T-cells have been shown to be less effective at tumour control *in vivo* [335]. Transduced T-cells could be phenotypically characterised *in vitro* before infusion into mice and during the experiment by isolating T-cells from tail bleeds. If transduced T-cells were characterised as terminally differentiated effector cells, efforts to reverse this phenotype could be employed. T-cells cultured *in vitro* in media containing IL7, IL12 and IL21 have been shown to revert to a less differentiated phenotype [336]. Therefore these cytokines could be utilised in culture prior to T-cell infusion to delay terminal effector differentiation. Johannessen *et al.* have investigated the use of IL7 and IL15 in culture media prior to the infusion of EBV-CTLs into SCID mice which have been injected with LCLs. The group demonstrated that mice which received CTLs cultured in this media survived longer than mice which received CTLs cultured in media which contained IL2 alone [325]. However the phenotype of the T-cells was not investigated, nor was the reason for the increased survival.

5.4.4 Conclusion

In conclusion, preliminary *in vivo* work performed here indicates that there may be tumour control by PRS-TCR transduced T-cells. These experiments require repeating and further optimisation, ideally infusing more engineered T-cells per mouse, to determine if the PRS-

specific TCR is capable of producing effective T-cell responses to control tumour outgrowth *in vivo.*

6 Discussion

TCR gene transfer is a form of immunotherapy that produces a specific immune response against a known target antigen. It has been designed for the treatment of multiple cancers and has shown promise in clinical trials [117, 139, 141]. This thesis focuses on TCR gene transfer for the treatment of EBV associated malignancies. As EBNA2 was identified in the majority of PTLD biopsies analysed here, and the EBNA2 derived epitope PRS is presented at high levels by MHC class II, TCRs against this epitope were isolated. A highly avid PRS-specific TCR was cloned, transduced into PBMCs and these transduced cells were shown to have therapeutic promise *in vitro.* This 'therapeutic promise' was demonstrated by the ability of the transduced T-cells to recognise and respond to target T-cells in a variety of assays. Preliminary *in vivo* studies showed that transduced T-cells most likely function to control tumour growth, however future work needs to be performed to optimise experiments in order to confirm this.

Here I will discuss the advantages of harnessing the CD4+ T-cell response for cancer therapy and in particular, the advantages of using MHC class II restricted TCRs in this T-cell subset. Additionally I will discuss further optimisation of TCR gene transfer therapy in the context of EBV associated malignancies. Finally, I will consider how TCR gene transfer therapy is likely to progress in the future.

6.1 PTLD treatment options

PTLD is a life threatening consequence of both HSCT and SOT. The current standard of care is Rituximab. As previously described, rituximab depletes all mature B-cells by targeting CD20. Response rates to this therapy are between 42%-70%, and so it is clear there are a

number of patients who do not respond. Furthermore, responders often relapse and this is associated with a high rate of mortality [213, 214, 218]. To overcome this, EBV specific CTLs are being investigated for the treatment of PTLD patients, with 51-67% overall response rates across a number of clinical trials [228]. Production of donor derived EBV-CTLs is time consuming and consequently third party EBV-CTLs are being generated, stored and given to partially HLA matched PTLD patients.

As not every patient will respond to Rituximab or EBV specific CTLs, other forms of salvage therapy are required. Transducing T-cells with an EBV specific TCR not only provides an additional therapeutic option for PTLD patients but it could do so cheaper than the EBV specific CTL alternative. Currently CTLs are generated and stored for long periods of time. This is costly and labour intensive and TCR gene therapy could overcome this. In addition, TCR gene transfer is an autologous therapy and so negates the risk of GvHD that is associated with adoptive transfer of third party EBV specific CTLs.

6.2 The anti tumour responses of CD4+ T-cells

CD4+ T-cells have been shown to be important in anti-tumour immunity. In a trial using EBV specific-CTL for the treatment of PTLD, a positive correlation was observed between clinical response and the frequency of CD4+ T-cells in the infused product [229]. Additionally, a T-cells product generated from TILs of which 95% of cells were CD4+ T-cells specific for a patient tumour-specific mutation resulted in tumour regression [81]. Furthermore, Merlo et al. have investigated the anti tumour effects of CD4+ versus CD8+ EBV specific-CTLs in a mouse model of human PTLD. Here, they found that CD4+ T-cells have therapeutic activity, even in the absence of CD8+ T-cells [323]. This CD8 independent

effect could be due to recruitment of other immune cell types, or could be a result of direct effector function of the CD4+ T-cells against the tumour.

As CD4+ T-cells can orchestrate a broad immune response *and* be directly cytotoxic, harnessing the response from this T-cell subset could be therapeutically beneficial. Harnessing the CD4+ T-cell response to EBV derived antigens is particularly useful, as CD4+ T-cells can directly recognise infected B-cells. This is due to endogenous viral antigens accessing the MHC class II pathway by mechanisms including autophagy, intercellular antigen transfer and other, as yet undefined pathways [33, 35]. We have shown here that CD4+ T-cells are capable of recognising physiological levels of antigen and responding by producing multiple cytokines, proliferating, maturing DCs and by killing target T-cells. These responses are all indicative of anti-tumour effects.

6.3 Transducing CD4+ T-cells with MHC class II restricted TCRs

6.3.1 Targeting MHC class II restricted antigens

one advantage of targeting MHC class II presented epitopes, is that tumours that have selectively down-regulated MHC class I as a form of immune evasion could be treated. It has been reported that 40-90% of human tumours down-regulate MHC class I, with the total number of MHC class I negative tumour cells within one biopsy being as high as 90% [57, 337, 338]. MHC class I loss can be reversible or irreversible, depending on the cause of the down-regulation. For example, loss of heterozygosity results in irreversible MHC class I loss whilst epigenetic modifications are reversible and can be treated with immunomodulatory cytokines such as IFNγ [57, 339, 340].

6.3.2 EBV-associated down-regulation of antigen presenting machinery

EBV has been associated with down regulation of several genes involved in MHC class I antigen presentation in NPC [341]. Although the EBV protein responsible for the loss of MHC class I antigen presentation genes in the context of NPC has not been elucidated, certain lytic proteins have been described as disrupting MHC class I antigen presentation. The late lytic protein BNLF2a has been described as preventing antigen presentation through MHC class I. BNLF2a prevents binding of peptides to TAP, which results in the inhibition of T-cell responses to immediate early and early lytic stage proteins [342]. Viral IL10 down-regulates TAP, reducing the quantity of peptides which are transported into the ER [343]. BGLF5 is an exonuclease which degrades host cell mRNA. As MHC class I and TAP transcripts are degraded, fewer MHC class 1 molecules are produced [344, 345]. BILF1 enhances MHC class I endocytosis and subsequent lysosomal degradation [346]. As many MHC class I restricted CD8+ T-cells are specific for immediate early and early EBV lytic antigens, EBV infected cells become protected from some CD8+ T-cell recognition[153].

There is limited evidence that EBV down-regulates MHC class II. LMP2a and BZLF1 have been reported to be able to down-regulate *MHC CLASS II* [347, 348]. However both LMP2a and BZLF1 are expressed (constitutively and sporadically, respectively) in LCLs, and CD4+ T-cells are able to respond to LCLs [153]. This suggests that any MHC class II down regulation by EBV is not sufficient to completely prevent T-cell responses. In addition, Stopeck et al. and Merlo et al. have shown that 94% of DLBCL cases (n=71) and 100% of PTLD cases (n=12) were MHC class II positive, respectively [323, 349]. Importantly, the results we have from PTLD biopsy staining suggest that MHC class II is still expressed on tumour cells when BZLF1 is expressed. Where studied, EBV associated malignancies

maintain MHC class II and therefore immune evasion from CD4+ T-cells by this method is unlikely.

6.3.3 Limitations of transducing MHC class I restricted TCRs into CD4+ T-cells

Transducing CD4+ T-cells with an MHC class II restricted TCR not only provides a therapeutic option for the treatment of tumours which have down-regulated MHC class I, but could also result in superior CD4+ T-cell responses, compared to if CD4+ T-cells were transduced with an MHC class I restricted TCR.

Although MHC class I restricted TCRs have been shown to be functional in CD4+ T-cells, producing IL2 and IFNγ in response to target T-cells, the functional avidity is often lower than that of CD8+ T-cells transduced with the same TCR, suggesting a level of CD8 dependence [237]. Here we have observed that CD8+ T-cells have a lower functional avidity than CD4+ T-cells when transduced with the same, MHC class II restricted TCR, supporting this concept of co-receptor dependence.

Co-transduction to express the CD8 molecule has been performed to rescue the lower functional avidity of CD4 + T-cells transduced with a MHC I restricted TCR [237]. However, due to the limitations of the amount of DNA which can be incorporated into one recombinant viral vector, co-receptors have to date been engineered into separate vectors [237]. GMP grade vectors are costly and therefore the addition of another vector to therapy would be expensive. Another consideration is that many more cells would need to be transduced to achieve the required number of co-transduced cells as the process will be less efficient. Furthermore there are already a number of other gene modifications proposed to improve safety and/or efficacy and so co-transductions may be better ‘reserved’ for these genes.

Transducing CD4 T-cells with a class II-restricted TCR overcomes this problem. Transducing CD4+ T-cells with an *MHC CLASS II* restricted TCR harnesses the optimal CD4+ T-cell response to tumours, whilst providing additional, MHC class II restricted targets.

6.4 Target Selection

6.4.1 Viral Antigens

Currently, identifying good epitope targets is limiting TCR gene therapy. Finding a target that is a) specifically expressed on tumour cells, b) shared between patients and c) required for cell survival and/ or proliferation and so unlikely to be 'lost' in order to escape immune destruction is a huge challenge.

Targeting viral antigens minimises on target off tumour toxicity as only EBV infected cells will express the target antigen, and these are infrequent, with only 1 in 10,000-50,000 circulating mature B-cells are infected [242].Therefore treating EBV associated malignancies by TCR gene transfer would remove the toxicities related with the current standard of care, Rituximab. Rituximab targets CD20, and consequently results in the destruction of all B-cells, regardless of their EBV status.

Additionally, viral epitope specific T-cells often have high avidity TCRs as they are not deleted during thymic tolerance. TCRs specific for shared tumour specific "self"-antigens are generally of weak avidity [350]. Whilst the avidity of these self-antigen-specific TCRs can be enhanced, for example by affinity maturation, there are safety concerns regarding the specificity of such manipulated TCRs [144].

6.4.2 EBNA2

One of the advantages of TCR gene transfer therapy is that it can be used in an 'off the shelf' manner, once TCRs against specific epitopes have been isolated. In order for such 'off the shelf' therapy to be clinically useful, it must have the ability to treat multiple patients. Therefore, epitope targets that are shared between patients must be identified. More than 200,000 cancers per annum are EBV associated and as such, EBV proteins are ideal targets for TCR gene transfer [351]. What's more, many EBV proteins are very immunogenic and as such, highly avid TCRs can be isolated. Specifically, EBNA2 is expressed in 75% of PTLDs tested here, 44% of AIDs related lymphomas and 28-32% of EBV positive DLBCLs, which are all MHC class II positive [247, 252, 267, 323, 349, 352]. Importantly, EBV has recently been identified as being present in malignancies previously thought to be EBV negative. These include up to 10% of gastric and an as yet an undefined percentage of breast cancers [353, 354]. Although the expression of EBNA2 is as yet unknown in these cancers, it is conceivable that in the future further EBV-associated tumours such as these may be identified that might be targeted.

6.4.3 Alternate MHC class II restricted antigen targets for the treatment of PTLD

As we have shown through immunohistochemistry, most PTLDs express EBNA2. Therefore many PTLD patients would benefit from a TCR gene transfer therapy that targets EBNA2 derived antigens. The PRS epitope is an ideal immunotherapy target as it is expressed at high enough levels on tumour cells for direct recognition by CD4+ T-cells [189]. Additionally, it is presented through a common HLA allele and so the number of patients that can be treated with this TCR is wide ranging.

However not all PTLD patients express EBNA2 and expression is limited in DLBCL and AIDs related lymphomas. Therefore targeting additional EBV proteins would provide a therapeutic option for a wider range of patients. Additionally, patients with the type 2 strain of EBV, Ag876, would not be able to be treated with this PRS-specific TCR, as this epitope is not present in this EBV strain. Although type 1 EBV is most common worldwide, the Ag876 EBV strain is just as common in sub-Saharan Africa [258]. Furthermore, as DR52b is present in approximately 40% of the Caucasian population, it would be beneficial to isolate a TCR against an epitope presented through a different HLA allele, in order to increase the percentage of patients who could be treated.

Currently there are few known MHC class II restricted antigens in comparison with those which are MHC class I restricted. However the importance of this T-cell subset in anti-tumour immunity is beginning to be realised, and efforts are currently on-going to identify more MHC class II restricted targets.

Several EBV latent protein derived epitopes that are presented through common class II HLA alleles have now been identified, including the EBNA1 epitope (TSL), the EBNA3c epitope (SDD) and the EBNA2 epitope (PAQ) [189]. T-cells against these targets have been shown to directly recognise naturally infected B-cells and therefore these are candidate epitopes for further analysis. They are presented through HLA DR103, HLA DQ5 and HLA DR14, respectively [189]. The www.allelefrequencies.net website shows that DR103 is present in low frequencies in the majority of world populations studied. DQ5 is present in over 50% of Chinese and Serbian populations and high in other populations. DR14 is present in a low frequency of the majority of the world populations investigated but is present in a high percentage of the Taiwanese population. Therefore cloning these TCRs could be particularly useful for different populations.

Additionally, epitopes derived from EBV lytic proteins have been shown to be recognised by CD4+ T-cell clones. Although lytic cycle protein expression is sporadic, these antigens are transferred between neighbouring cells to be presented through MHC class II. Therefore antigen negative tumour cells can be targeted for destruction by CD4+ T-cells when antigen is transferred from tumour cells expressing lytic proteins [188]. As we have shown in the PTLD biopsies analysed here, BZLF1 is expressed in some tumour cells, and so work could be continued on the isolation of BZLF1 specific TCRs for the treatment of PTLD.

Interestingly, Long et al have described CD4+ T-cell clones isolated from EBV seronegative people, which respond to LCLs and a range of tumour cell lines. The clones produce IFNγ in response to target T-cell lines and also have direct effector responses, killing target T-cells in both short and long term assays. Recognised tumour cell lines include HLs, BLs, follicular lymphoma, T-lymphoma and multiple myeloma. The target epitopes were not from EBV lytic or latent proteins and so considered to be of cellular origin. Whilst the specific target epitopes of these T-cells are still under investigation, the clones did not respond to a panel of autologous non-transformed cell lines (activated B-and T-cells and DCs), indicating that the target epitopes may be up-regulated or only expressed in transformed cells [329]. Similarly, Linnerbaur et al have identified CD4+ T-cell clones with unknown specificities [236]. They too found CD4+ T-cell clones which produced cytokines and cytolytic responses towards LCLs and did not appear to be restricted to any EBV antigens. If the epitopes are identified and shown to be not expressed in other healthy tissues, TCR gene transfer with the TCRs from these clones could provide a therapy for the treatment of a wide range of lymphomas.

6.5 Future MHC class II restricted TCR gene transfer targets (general)

For MHC class II restricted target selection of a broader range of tumours, Sahin et al have used next generation sequencing of tumours to identify patient specific neo-antigens, which are predicted to be processed and presented in MHC class I and II. Neo antigens were identified in all cancers analysed; namely melanoma, colon and breast cancer. Interestingly, 80-90% of predicted neo-antigens were recognised by CD4+ T-cells [355]. In mouse studies, vaccinating against neo-antigens present in melanoma, colon and breast cancer cell lines show that CD4+ T-cells are capable of controlling tumours and prolonging mouse survival. Through epitope spreading, this CD4+ T-cell response can also lead to induction of CD8+ T-cell responses. Not only does this work highlight the anti-tumour effects of CD4+ T-cells but it provides evidence that there are a large number of MHC class II restricted antigens which could in the future be used for TCR gene transfer therapy.

Shared mutations would need to be identified for TCR gene transfer therapy at present; however the future may provide the technological advancements needed to treat patients individually, according to their specific neo-antigens.

6.6 TCR gene transfer optimisation to treat EBV associated malignancies

TCR gene transfer has shown clinical success in some settings. There has however, been much work to further optimise this approach. Here I will discuss ways in which TCR gene transfer for the treatment of EBV associated malignancies could be further improved.

6.6.1 Resistance to Immunosuppression

In the context of transplant patients, it would be beneficial to engineer T-cells to render them resistant to immunosuppression. Transplant patients receive immunosuppression to prevent graft rejection, and this treatment could inactivate or delete infused T-cells, preventing therapeutic effects. Generating immunosuppression resistant T-cells has been achieved in pre-clinical studies. Riciardelli et al have shown that T-cells can be successfully engineered with a calcineurin mutant to render them resistant to the immunosuppressive drug tacrolimus [232]. By engineering T-cells in this manner, transplant patients could be administered T-cells without compromising the levels of immunosuppression given. As reduced immunosuppression often results in graft rejection, this could be prevented. The group have developed a strategy to rapidly engineer EBV-CTLs to be tacrolimus resistant and this therapy will be tested in a clinical trial [231, 232].

6.7 General TCR gene transfer optimisation

6.7.1 T-cell differentiation

The differentiation status of infused T-cells has been shown to have an effect on anti-tumour responses. Conventionally, T-cell based immunotherapies have relied upon *in vitro* expansion of antigen specific target T-cells, or T-cell activation for successful transduction. Activating and expanding T-cells drives their differentiation into a terminal differentiated effector phenotype. These cells have been shown to have limited persistence, limiting their therapeutic effectiveness. Therefore efforts have gone into determining the ideal T-cell phenotype for immunotherapy and the generation and/or maintenance of such phenotype. Naive T-cells have been shown to have great therapeutic potential, as they have a high proliferative capacity and have a survival advantage over effector cells [356]. Frumento et

al. have used cord blood T-cells as a source of naïve T-cells and report that cord blood derived T-cells transduced with an EBV specific TCR maintained a less differentiated phenotype and longer telomeres than transduced T-cells derived from adult blood [241]. The limitation of this approach is that cord blood T-cells are not autologous and so could theoretically cause GvHD.

The functional benefit of adoptively transferring stem cell memory T-cells (Tscm) has been demonstrated. In animal studies Tscm have been shown to expand better than any other T-cell subtype and persist even in the absence of antigen [357, 358]. Interestingly, experienced T-cells can be reverted back to Tscm cells. Telomere lengths in these reverted T-cells have been shown to be long, and the cells can expand better than effector cells, do not express exhaustion markers and are more cytotoxic [359]. It is likely that future trials with adoptively transferred T-cells will aim to induce Tscm phenotypes.

Whilst transduction of multiple genes is required to generate induced Tscm T-cells, the generation of other T-cell phenotypes is much simpler [359]. The *in vitro* conditions in which T-cells are cultured affect the T-cell differentiation status. IL15 and IL7 have been proven to enhance T-cell survival by maintaining a more naive phenotype, and IL21 has been used in combination with IL15, and has been shown to synergistically enhance T-cell effector functions [360, 361].

6.7.2 Combinational therapy

The clinical successes of checkpoint blockade inhibitors, including anti-PD1 and anti-CTLA4 monoclonal antibodies, has taught the field much about anti-tumour responses. Firstly, success of these therapies highlights the power of the immune system when inhibition is removed. Secondly, as the types of tumours which are treated with these therapies are constantly increasing, they have emphasised how many tumours employ such immune suppressive techniques. Interestingly, the biggest successes with these therapies occur in tumours with high mutational loads, such as melanoma and NSCLC [362, 363]. This shows that many patients with these tumours have T-cells which are able to target them; however they are not able to function in the face of such suppression. Combination therapy of PD1 blockade and TCR gene transfer therapy may be advantageous to enhance activity of the transferred T-cells in immunosuppressive tumours.

Indeed, by combining PD-L1 blockade with adoptive T-cell therapy in a mouse squamous cell carcinoma model, Strome et al have demonstrated a synergistic effect, with a survival advantage observed in mice which received both T-cells and anti PD-L1 [364]. In addition, it has been shown that by blocking PD1, T-cells have enhanced ability to migrate to the tumour in a chemokine dependent mechanism [365]. Most importantly, Abate-Daga et al have analysed the phenotypes of T-cells that have been transduced with tumour specific TCRs and administered to patients with melanoma. T-cells were isolated from peripheral blood samples one month after infusion and a gene array was performed, to compare the phenotype of these T-cells with the pre-infusion product. Results showed that of 511 immune related genes analysed, 156 were differentially expressed following infusion. Specifically, PD1 was up-regulated. This up-regulation correlated with reduced IFNγ production when cells were co-cultured with PD-L1 positive target T-cells, compared to the response observed from pre-

infusion T-cells [366]. There is currently a trial on-going which will look at the combination of anti-CTLA4 blockade and autologous tumour specific CD8+ T-cells for the treatment of metastatic melanoma (NCT02027935).

In the context of PTLD, some EBV positive cases have been shown to be PD-L1 positive by IHC (19/26 cases) [328]. Additionally, 27-100% EBV+ DLBCL samples have been shown by IHC to express PD-L1 and 28% AIDs related lymphomas [367, 368]. Together, this suggests that at least some patients with these malignancies may benefit from combinational therapy combining TCR gene transfer and PD1 or PD-L1 blockade.

Further combination therapies are being investigated in pre-clinical and clinical studies, with emphasis on combining chemotherapy with immunotherapy. Chemotherapy is used routinely prior to adoptive T-cell therapy for lymphodepletion. This creates 'space' in the hematopoietic system for expansion of transferred T-cells by homeostatic proliferation, increasing the amount of activatory cytokines available to the transferred T-cells and removing suppressive T-cells [369].

Other than creating a T-cell proliferative environment, chemotherapy has been shown to enhance T-cell responses through additional mechanisms. For example, consistently giving a low dose of cyclophosphamide to end stage, chemotherapy resistant patients, selectively suppresses and depletes Tregs without affecting other immune cells [370]. Through this action, T-cells and NK cells proliferate and function more effectively [371]. In addition, gemcitabine can eliminate immunosuppressive myeloid derived suppressor cells (MDSCs), without inhibiting other immune cells [372]. Importantly for CD4+ T-cell mediated immunotherapy, Ding et al have highlighted the effects of chemotherapy on this T-cell subtype. The group administered a single dose of cyclophosphamide to B-cell lymphoma bearing mice, before transferring tumour specific CD4+ T-cells. This pre-treatment prevented

PD1 expression on CD4+ T-cells and resulted in improved cytokine production, proliferation and anti-tumour immunity, compared to control mice [373]. As some PTLDs have been shown to express PD-L1, combination therapy of chemotherapy and TCR gene transfer to target PTLD could be beneficial.

Finally, as TCR gene transfer provides T-cell responses against individual epitopes, combining TCR gene transfer therapies could be investigated. This would reduce the risk of the therapy failing due to epitope loss. EBNA3b has been reported to be mutated in a patient following EBV-CTL infusion for the treatment of PTLD. The mutation was a deletion, of which the deleted region contained two CTL epitopes. As these two epitopes were the predominant target of the infused T-cells in this patient, the CTLs were less responsive to the tumour. The result of this deletion was that EBNA3b mutated tumour cells continued to proliferate and tumour progression resulted in patient death [374]. We have not investigated epitope loss as part of this work but future work could address this. If any tumours remain in mice after treatment with PRS-TCR transduced T-cells, they could be resected and analysed by IHC for EBNA2 expression in the tumour cells. This could be compared to results obtained from mice treated with mock transduced T-cells to demonstrate if antigen loss occurred. *Ex vivo* tumours could also be sequenced to determine if the PRS epitope is present in the tumour sample, or if, like the case of EBNA3b mutation, this critical region is lost in order to escape immune response.

Additionally, by combining MHC class I and MHC class II TCR gene transfer therapies, optimal CD4+ and CD8+ T-cell responses could be harnessed. Our group has previously generated a HLA A11 restricted LMP2 TCR that was shown to function effectively *in vitro* and *in vivo*, producing cytokines and cytotoxic responses to target T-cells [235]. This could be used in combination with the TCR described here to investigate if targeting epitopes from

different proteins with different HLA restrictions results in an improved anti-tumour response. It has been noted by others that when anti-tumour CD4+ T-cells are functional *in vivo,* they mediate epitope spreading [355]. If CD4+ T-cells transduced with a PRS specific TCR could mediate epitope spreading, non-transduced T-cells could be activated and aid tumour clearance, even in the face of epitope loss.

6.8 Future Developments in TCR gene transfer therapy

6.8.1 Targeting non-hematopoietic tumours with MHC class II restricted TCRs

MHC CLASS II is constitutively expressed in professional APCs. MHC class II can be conditionally expressed in other cell types. As such, MHC class II restricted TCRs may in the future be used to treat tumours which are classically *MHC CLASS II* negative. MHC class II expression can be induced with IFNγ [375-377]. However all patients treated systemically with IFNs have suffered fever, chills, tachycardia, malaise and headaches. In addition, gastrointestinal symptoms, neurotoxicities and hematologic toxicities are common [378]. Therefore several ways to limit these toxicities have been explored.

Zhang et al have transduced TIL products with a plasmid containing an inducible cytokine, IL12 [379]. Expression of these cytokines is dependent upon NFAT signalling, as NFAT response elements are contained within the promoter. When transduced cells are activated through TCR signalling, NFAT is expressed and the expression of the co-transduced cytokine is induced. This selective cytokine expression overcomes the limitations of systemic cytokine administration. A phase I clinical trial treated metastatic melanoma patients with TILs transduced with this construct has shown that 10/16 patients achieved short lived objective responses [380]. However, at doses greater than $3x10^9$ cells, serious adverse events were

recorded. Toxicities included liver dysfunction, fever and hemodynamic instability. As T-cells are being administered to patients pre-conditioned by chemotherapy and irradiation, there is space for T-cell homeostatic proliferation. NFAT drives T-cell proliferation, and so it is conceivable that NFAT is activated during homeostatic proliferation, leading to systemic expression of IL12 [381, 382].

An alternative method to selectively up-regulate cytokines in the microenvironment of solid tumours would be to induce expression in hypoxic regions. Due to disrupted angiogenesis in solid tumours, hypoxia is common [383]. This hypoxia could be harnessed therapeutically by generating a construct which contains a cytokine that is expressed when HIF is up-regulated, by engineering HRE repeats into the promoter. HRE driven gene expression has been performed by Dachs et al and shown to work effectively [384]. As part of this thesis I commenced work to generate such construct to drive the expression of IFNγ and other cytokines, after confirming by western blot that HIF is up-regulated in T-cells in hypoxic conditions. However, due to technical challenges and time limitations, this work was discontinued.

6.9 Large Scale Production of T-cell products

Engineering of T-cells has shown unprecedented success in phase I clinical trials, with clinical responses observed in advanced cases that have failed conventional treatments. Nevertheless, current approaches are complex and costly to deliver. Therefore, there is currently much interest in making this approach more widely available. Importantly, technological advancements have allowed for widespread use of cell products for HSC transplants and so there is no reason why advancements within T-cell therapy will not happen over the next few years. Indeed, much technological advancement in T-cell manufacturing

has already occurred. That pharmaceutical industries are showing interest in CARs hints that genetically engineering T-cells for adoptive therapy is now considered feasible and likely to enter mainstream cancer therapy.

6.9.1 T-cell manufacturing

To minimise the amount of handling of T-cells, G-REX flasks and WAVE bioreactors are now commonly used for generating T-cells to treat patients [385]. These flasks allow for rapid expansion of T-cells and media is continually perfused in a closed environment, complying with Good Manufacturing Practice (GMP) regulations [386]. T-cells are still ficoll separated from PBMCs in open systems, and so a process in which T-cells can be obtained from peripheral blood and cultured *ex vivo* in a fully closed system is desirable.

If gamma retroviruses are being used, sTable virus producing cell lines are amplified in bulk and one virus stock can be harvested for up to three days. On the other hand, lentivirus production is more complicated, as sTable lentivirus producing cell lines are difficult to generate. Additionally, the removal of cellular contaminants after virus production and the concentration of virus whilst maintaining potency is complicated[387].

To minimise the number of highly skilled workers and specialised laboratory facilities required to generate these T-cell products it is more efficient to use specialised centres for manufacturing and then ship the T-cell product to the patient. A similar approach has already been used successfully with third party EBV-CTLs for the treatment of partially HLA matched PTLD patients [212, 230, 388].

6.9.2 Universal T-cells

TCR gene transfer is complicated by the need to use patients own T-cells, as isolating individual patient T-cells is labour intensive. In an attempt to generate an 'off the shelf' approach, 'Universal T-cells' are currently under investigation. Like 3rd party EBV-CTLs, these would be generated and stored for use in an allogeneic recipient. As well as negating the need to isolate T-cells from each patient, universal T-cells could be prepared in bulk, removing the complexity of individual T-cell preparation and reducing waiting times for therapy. This would make the T-cell preparation and manufacturing process cheaper and simpler. Endogenous TCRs are knocked down by zinc finger nucleases in 'Universal T-cells' to prevent GvHD and improve exogenous TCR expression and pairing [389]. Furthermore, HLA genes are being knocked down to prevent poor T-cell persistence due to graft rejection [390]. Such HLA negative T-cell could generate NK responses, and so to overcome this, non classical HLA expression could be enforced in these cells [391].

6.9.3 Cost

A significant barrier to the wider application of T-cell therapies is cost. As discussed, preparation of therapeutic cell products is complex and cell products are currently produced as patient-specific products. This will clearly result in high preparation costs, but whether these costs are higher than the costs of conventionally generated molecular products is questionable. The cost of TCR gene transfer therapy has been estimated to be $15,000 - $25,000 per patient [392]. Whilst this estimation does not include overhead and rental costs, it is clearly much less than some FDA approved monoclonal antibodies. For example, Ipilumumab costs over $100,000 per year per patient, and has shown limited responses [393].

Costs could be reduced in the future by transducing cells by non-viral means rather than with retro-or lentiviruses. The DNA plasmid Transposon-transposase systems such as Sleeping Beauty are electroporated into target T-cells require GMP grade plasmids rather than viruses and can accordingly be produced and purified easily and relatively cheaply [136]. Also they can carry up to 10 kilo-base pairs of DNA. This is far more than a virus could carry and as such, these transposon systems could transfer multiple genes in one plasmid [136]. This would make significant cost savings by avoiding the need for multiple vectors. However at present transduction is inefficient using these systems so further development is required before they replace virus-based systems [138].

6.10 Conclusion

This thesis investigates the therapeutic potential of MHC class II restricted TCR gene transfer for the treatment of PTLD and other EBV associated malignancies which express EBNA2. CD4+ T-cells which recognised the EBNA2 derived antigen PRS, or the BZLF1 derived antigen LTA, through the common MHC class II allele, DR52b, were isolated and cloned. Clones were subjected to a range of assays to determine which had the greatest potential to be effective therapeutically. After testing CD4+ T-cell clones for their response to epitope peptide and target T-cells expressing naturally processed and presented antigen, promising clones were selected for further analysis. The clone with the highest functional avidity that was confirmed to be restricted through DR52b and proven to be monoclonal was selected for TCR isolation. Here, BZLF1 specific TCRs were dropped as no T-cell clone identified in this body of work was suiTable for continuation. Specifically, only one LTA specific clone expanded to sufficient levels for *in vitro* functional analysis, and this was shown to be oligoclonal by DR52b-LTA tetramer staining.

Focus thus shifted onto the therapeutic potential of the PRS specific TCR which was isolated from the selected CD4+ T-cell clone (clone 93) and transduced into healthy donor PBMCs. *In vitro* functional studies of transduced CD4+ and CD8+ T-cells showed that the TCR genes were translated, alpha and beta chains paired correctly and the introduced TCR complex was efficiently trafficked to the cell surface. Transduced cells had a high functional avidity, which was similar to the functional avidity of the parent clone. Interestingly, CD4+ T-cells had a higher functional avidity than CD8+ T-cells, suggesting a degree of CD4 co-receptor dependency. CD4+ T-cells maintained their helper function, as was evident by their ability to mature DCs. Most importantly, transduced T-cells were able to recognise target T-cells expressing physiological levels of antigen and responded by proliferating, producing multiple cytokines and killing.

In vitro functional studies suggested that the transduced T-cells would be able to control tumour *in vivo*. A mouse model of PTLD was established which comprised of immunocompromised mice which were administered LCLs. After four days, mice were treated with transduced or mock T-cells. Results hinted that the transduced T-cells have some control over tumour growth, yet statistical significance was not consistently reached in these preliminary experiments. Unfortunately time constraints limited the *in vivo* work performed here, however future experiments could be set up to more fully investigate the function of T-cells transduced with an MHC class II restricted PRS TCR *in vivo*.

Finally, the work performed here provides evidence that the transfer of MHC class II restricted TCRs is viable and effective. Whilst this work could be continued to prepare such therapy for clinical trial for the treatment of PTLD, it also acts as a model for harnessing CD4+ T-cell responses. The lessons learned in this system could be applied to any other model where stimulating CD4+ T-cell responses would be advantageous.

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